

# INVESTIGATING THE ROLES OF NOTCH SIGNALLING DURING INNER EAR DEVELOPMENT: NEW INSIGHTS INTO THE DYNAMICS OF NOTCH ACTIVITY AND THE FUNCTIONS OF DELTA1

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Elena Chrysostomou

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# DECLARATION

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I, Elena Chrysostomou, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in this thesis.

# ABSTRACT

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The Notch signalling pathway has ubiquitous roles during inner ear development. An early phase of Notch activity, via the ligand Serrate1 mediates lateral induction and promotes the early formation of inner ear sensory patches. In addition, Notch signalling mediated by the ligand Delta1 regulates otic neurogenesis and hair cell formation by lateral inhibition.

To get new insights into the dynamics of Notch-activity during these processes, a fluorescent reporter consisting of the *cis*-regulatory element of the mouse Hes5 gene was introduced in the embryonic chick inner ear. In *ovo* electroporation was used to transfect the otic cup and to demonstrate the sensitivity of the Hes5 reporter to Notch activity. Using the retroviral vector RCAN and the Tol2 transposon system this reporter was stably integrated into the cells genome following electroporation of the otic cup. At late stages of inner ear development, the reporter was specifically activated in sensory progenitors and supporting cells but not in differentiated hair cells. The reporter was also shown to be sensitive to artificial gain- and loss-of-Notch activity. Time-lapse imaging of transfected sensory epithelia showed intercellular differences in fluorescent levels and variations over time suggesting endogenous variations of Notch activity occur within progenitor cells. Extensive proliferation and cell rearrangements could also be directly visualised at the time of hair cell differentiation.

In order to test the role of lateral induction during sensory patch formation, the Hes5 regulatory element was next used to drive Delta1 expression within Notch-active cells. This created an artificial positive feedback loop mimicking the endogenous lateral induction via Serrate1. This resulted in abnormal inner ear morphogenesis and disrupted hair cell differentiation within the sensory epithelia. The inner ears transfected with the Hes5-Delta1 construct had a smaller vestibular region, cochlear defects and ectopic hair cell formation, suggesting abnormal boundary formation between sensory and non-sensory regions. Furthermore expression of Delta1 inhibited hair cell formation in *trans* but promoted hair cell differentiation in *cis*. The analysis of these effects at the single cell level provided new insights into the function of Delta1 during lateral inhibition.

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# DEDICATION

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To my wonderful family; Dad, Mum, Alex and Harris

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# ABBREVIATIONS

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AP	anterior-posterior
bp	basilar papilla
CBF	C-promoter binding factor
CBS	CBF binding site
CSL	CBF-1 in mammals, Su(H) in <i>Drosophila</i> , Lag-1 in <i>C.elegans</i>
CVG	cochlea-vestibular ganglion
DAPT	N-[N-(3, 5-difluorophenacetyl)-1-ala-nyl]-S-phenylglycine t-butyl ester
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DSL	Delta/Serrate/Lag2
DV	dorsal-ventral
E	Embryonic day
EGFP	Enhanced Green Fluorescent Protein
GER	greater epithelial ridge
HES	Hairy and Enhancer of Split
HH	Hamburger Hamilton
IHC	inner hair cell
kb	kilobases
LER	Lesser epithelial ridge
MAM	mastermind
MET	mechanoelectrical transduction
ML	medial-lateral
NICD	Notch intracellular domain
NLS	nuclear localisation signal
OHC	outer hair cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	paraformaldehyde
RCAN	<u>R</u> eplication <u>C</u> ompetent <u>A</u> SLV LTR with <u>no</u> <u>S</u> plice acceptor site
RCAS	<u>R</u> eplication <u>C</u> ompetent <u>A</u> SLV LTR with a <u>S</u> plice acceptor site
RNA	ribonucleic acid
ROI	region of interest
SuH	Suppressor of Hairless
YFP	Yellow Fluorescent Protein

# 1

## INTRODUCTION

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This introduction will describe the basic anatomy and function of the inner ear, inner ear development and the Notch signalling pathway.

### 1.1 Hearing and sound

The ear is divided into three main compartments: the outer, middle and inner ear (Figure 1.1). Sound first arrives at the outer ear, which consists of the pinna and the ear canal (auditory meatus). Sound can either enter the ear canal directly or can be reflected from the pinna. The pinna, due to its large surface area, is designed to direct more sound to the ear canal than would otherwise arrive. As well as directing the sound, the pinna modifies sounds to aid vertical localization. The ear canal connects the pinna to the eardrum (tympanic membrane) and it is involved in the amplification of sound at speech frequencies (250 Hz to 4 kHz). The middle ear consists of the tympanic membrane and three small interconnected bones; malleus (connected to the eardrum), incus and stapes (connected to the oval window of the cochlea). The function of the middle ear is to translate the energy of air pressure waves into mechanical vibrations. Then the cochlea, within the inner ear, propagates these mechanical signals as waves in fluid and membranes. These are then converted into nerve impulses that are transmitted to the brain.

### 1.2 The mammalian inner ear

“One of the most remarkable displays of precision microengineering in the vertebrate body” (Swanson et al., 1990).

The inner ear is a complex organ with an elaborate three-dimensional structure. In humans, the inner ear is located within the hardest bone in the

body, the temporal bone, making this organ difficult to access. In lower vertebrates it is contained within an auditory bulla that can be relatively easily separated from the skull. The inner ear is composed of a series of interconnected membranous tubes (the membranous labyrinth) that are encased inside bony channels which are positioned at the base of the skull (Forge and Wright, 2002). The fluid within these tubes is called endolymph and has a different ionic composition to the perilymph, the fluid that lies within the bony channels. The composition of the perilymph is similar to extracellular fluid; a high sodium ion ( $\text{Na}^+$ ) /low Potassium ion ( $\text{K}^+$ ) composition. Conversely, the endolymph has a high potassium ion concentration  $\text{K}^+$  (140 mM) and a low sodium ion concentration. It is essential for the normal functioning of the inner ear that these two fluids remain separated by the different types of epithelia that surround the endolymphatic compartment: the sensory epithelium, ion transporting epithelia and other unspecialised epithelia.

The membranous labyrinth consists of six anatomically separate mechanosensory epithelia belonging to the six sensory organs of the mammalian inner ear. The vestibular system consists of three ampullae of the posterior, superior and lateral semicircular canals, which house the sensory cristae and two separate macular epithelia of the saccule and the utricle. These are responsible for detecting angular and linear acceleration, respectively. These functions are necessary to detect the body's position in relation to gravity and to ensure balance and equilibrium. The sensory epithelium of the cochlea responsible for detecting sound is named the organ of Corti in mammals. The sensory organs receive afferent and efferent innervation from the VIIIth cranial nerve.

**Figure 1.1 Gross anatomy of the human ear.** There are 3 main compartment to the ear. The outer ear which consists of the pinna and the ear canal (auditory meatus), the middle ear which consists of three ossicles: the malleus, the incus and the stapes and the inner ear which consists of the vestibular organs (3 cristae, saccule and utricle) and the auditory organ (cochlea) The semicircular canals project from the dorsal region of the inner ear (Adapted from Seewald and Tharpe (2011)).

### **1.2.1 The cochlea**

The primary auditory sensory organ, the cochlea, has a snail-like morphology in mammals and is located in the ventral region of the inner ear. In large animals, it can be greater than 60 mm long and the width of the sensory epithelium can be up to 100  $\mu\text{m}$ . The human cochlea is approximately 30-35 mm long. Within the cochlea lies a collagenous basilar membrane, which begins narrow and thin at the basal end and increases progressively in width and thickness towards the apex. A cross section of the cochlea shows that it is divided into three separate chambers: the scala vestibuli and the scala tympani, which are filled with perilymph and the scala media, which is filled with endolymph (see figure 1.2A). The maintenance and circulation of these fluids are critical for cochlear function. Potassium ions are pumped into the scala media by cells of the stria vascularis, which lies against the lateral wall of the cochlea duct. The ionic difference provides the driving force for mechanoelectrical transduction. The scala media has an electrical potential of  $\sim 80\text{mV}$  compared with the scala vestibuli and tympani which is  $0\text{mV}$  (the endocochlear potential). Endolymph is separated from the perilymph in the scala vestibuli by the Reissner's membrane and in the scala tympani by the organ of Corti and the adjacent non-sensory epithelium. The scala vestibuli is continuous with the scala tympani at an opening at the apex of the cochlea called the helicotrema.

The organ of Corti, similarly to other sensory epithelia of the inner ear, is composed of mechanosensory hair cells and associated non-sensory supporting cells. They are arrayed in regular rows that extend along the length of the cochlear duct (See figure 1.2B). The human cochlea consists of  $\sim 15,500$  hair cells (3,500 inner hair cells (IHC) and 12,000 outer hair cells (OHC)). The IHCs are the primary sensory receptors and are arranged in one row along the inner edge of the organ of Corti. They are innervated by the primary sensory afferent neurons in the spiral ganglion. They receive  $\sim 95\%$  of the afferent innervation. The OHCs have a cylinder shape and are arranged in three rows along the outer edge of the organ of Corti. They only receive  $\sim 5\%$  of the afferent innervation, and receive mainly efferent innervation by fibers from the superior olive of the brainstem. The function of OHC is to modulate and sensitise IHC function. IHC are separated from OHC by pillar cells, which form the tunnel of Corti.



**Figure 1.2 Cochlear anatomy (A) Cross-section of the cochlea (B) The organ of Corti.** The organ of Corti consists of one row of inner hair cells and three rows of outer hair cells which are separated by the tunnel of Corti formed by the inner pillar cells and the outer pillar cells. The inner hair cells are separated from one another by the inner phalangeal supporting cells and the outer hair cells are separated by Deiters' cells. Border cells are located medial to inner ear hair cells and Hensen's and Claudius' cells are located lateral to outer hair cells (not shown) (adapted from Kelley (2006) and Encyclopaedia Britannica, Inc 1997).

Stereocilia are plasma membrane bound finger-like projections enclosing filaments of the cytoskeletal protein, actin, cross-linked with fimbrin and other actin-binding proteins. The hair bundle is composed of rows of stereocilia that increase in height and a single kinocilium located behind the row of longest stereocilia. The kinocilium is a true cilium, similar to motile cilia, which disappears from auditory hair cells as they mature. Adjacent stereocilia are connected by short extracellular links, which maintain the integrity of the bundle (Forge and Wright, 2002). Tip-links are connected from the tips of the shorter stereocilia to the shafts of the longer adjacent stereocilia (Gillespie et al., 2005). Deflection of the bundle towards the tallest stereocilia opens the mechanoelectrical transduction (MET) channels. A structure called the tectorial membrane forms a roof over the organ of Corti. It is composed of a gel-like connective tissue in which OHC, but not IHC, stereocilia are embedded. The tectorial membrane is important for hair cell mechanosensory transduction (see below).

The sensory epithelium converts mechanical energy (fluid motion in the inner ear induced by sound vibrations or head movements) into neuronal impulses, a process known as mechanotransduction. The stereocilia protrude into the fluid filled cavities of the inner ear and are deflected in response to vibrations. A deflection in the positive direction (towards the tallest stereocilium) leads to the opening of mechanoelectrical transduction (MET) channels, located on the stereocilia. The MET channel is a non-selective cation channel that allows the flow of potassium ions ( $K^+$ ) into the cell. Calcium ions ( $Ca^{2+}$ ) can also enter the MET channel which are important for sensory adaptation and for active mechanical responses in the hair bundle (Kennedy et al., 2005). There is a large electrochemical gradient between the endolymphatic fluid (+80mV) and the hair cell cytoplasm (-50mV) which drives the flow of positively charged  $Ca^{2+}$  and  $K^+$  ions through the MET channels during stimulation. This influx of positively charged ions depolarises the hair cell, which then activates voltage-gated calcium channels, leading to the release of the glutamate neurotransmitter at its synaptic pole. IHCs receive ~20 afferent endings each, but one afferent fiber innervates one hair cell only.

OHCs possess the same MET machinery but they also express a protein called prestin in their membrane which is sensitive to changes in membrane potential, leading to a conformational change that forces cell length changes at acoustic frequencies (Dallos et al., 2006). Hair bundles can also generate mechanical forces (Kennedy et al., 2005) and the two mechanisms together

are thought to be responsible for the amplification and tuning of the mechanical responses of the basilar membrane. This amplification enhances human hearing sensitivity by 40-60dB (Liberman et al., 2002). OHCs receive primarily an efferent innervation, mediated by acetylcholine receptors, which leads to calcium influx and activation of calcium-activated potassium channels. The innervation to the hair cells passes along the bony spiral lamina and into the Rosenthal's canal, where the spiral ganglion is located. The spiral ganglion includes all the cell bodies of the primary sensory neurons whose axons project via the VIIIth cranial nerve that transmits signals to the cochlear nuclei in the brainstem.

### **1.2.2 Pathology and treatment of hearing loss**

Auditory hair cells are highly susceptible to intense noise, ototoxic drugs, infections (meningitis, measles rubella and mumps) and aging as well as genetic defects. Hair cell loss in humans and mammals is irreversible, and their absence in the organ of Corti or the vestibular organs causes deafness and balance problems, respectively. Deafness is one of the most widespread disabilities: approximately 25% of the world's population have hearing difficulties with sensorineural deafness being the most common form of hearing loss.

Non-mammalian vertebrates such as birds (Corwin and Cotanche, 1988; Cotanche et al., 1994; Cruz et al., 1987; Ryals and Rubel, 1988) fish (Popper and Hoxter, 1984) and amphibians (Baird et al., 1993) have the ability to regenerate their hair cells after injury and thereby restoring sensory function (reviewed in Stone and Rubel, 2000). There is also evidence of vestibular hair cells being replaced in the mammalian inner ear (Forge et al., 1993; Forge et al., 1998; Li and Forge, 1997; Warchol et al., 1993) however this process is relatively slow and there is no evidence of replacement in the organ of Corti (Forge et al., 1998).

There are different therapeutic approaches for replacing hair cells being investigated such as stem cell therapy and inner ear gene therapy (Matsui et al., 2005). Because the basic rules controlling hair cell production are likely to be conserved during development and regeneration, understanding the mechanisms of hair cell formation during development has been the focus of intense research over recent years. Research in this area has already informed us on the therapeutic approaches to regeneration. *Atoh1* gene delivery through adenoviral vectors was the first study to demonstrate

cellular and functional repair in the organ of Corti of mature deaf mammals (Izumikawa et al., 2005).

### **1.2.3 *The chicken as a model organism to study inner ear development***

The avian inner ear consists of eight sensory organs. The vestibular sensory organs include three cristae and four maculae of the utricle, saccule, lagena and neglecta (Kido et al., 1993; Landolt et al., 1975). The macula neglecta is the smallest of the eight sensory organs and is located on the floor of the utricle immediately anterior to the posterior crista ampullaris (Correia et al., 1974).

The avian auditory sensory epithelium, the basilar papilla, is located within the cochlear duct. It differs greatly from the mammalian cochlea, in that instead of it being a coiled tube, it is an elongated, slightly curved tube (Figure 1.3). The basilar membrane with hair cells runs along its length. There is a graded increase in hair cell height from the inferior to superior edges. There are three types of hair cells: tall, intermediate or short depending upon their height (Takasaka and Smith, 1971). The general topology of the basilar papilla is similar to the mammalian cochlea. There are three scalae, tympani, vestibuli and media, present together with a tectorial membrane that overlays the sensory hair cells. At the distal portion of the basilar papilla is the macula lagena (Manley et al., 1991), which is unique to amphibians and birds. The lagena is a U-shape band of sensory hair cells and support cells and it is covered with otoliths much like the vestibular organs. Its function is currently unknown, but new insights indicated that the avian lagena has a vestibular nature (Galiccia et al., 2010). Its otoliths have high iron content, suggesting that this organ may be implicated in the reception of magnetic cues by birds during navigation (Harada et al., 2001).

**Figure 1.3 The developmental time series of chick and mouse inner ear morphogenesis.** Lateral views of paint-filled membranous labyrinths. Abbreviations: aa, anterior ampulla; asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; ed, endolymphatic duct; es, endolymphatic sac; la, lateral ampulla; lsc, lateral semicircular canal; pa, posterior ampulla; psc, posterior semicircular canal; s, saccule; u, utricle, D, dorsal; A, anterior. Scale bar, 200  $\mu\text{m}$ . Adapted from Cantos et al. (2000) and Brigande et al. (2000b)

The chick embryo is an excellent model for studying the molecular mechanism underlying early embryogenesis and development. Fertilised chicken eggs are available all year round, they are inexpensive and a specific quantity can be purchased which prevents excess and shortfalls. Fertilised eggs start developing when incubated at 38°C but can be stored in a cool place until they are required (but not for longer than a week) allowing for flexibility in experiments. The most comprehensive published stage series is the Hamburger and Hamilton stage series, which covers the 21 day gestation period and consists of detailed descriptions and accompanying photographs of the chick embryos at 45 different stages (Hamburger and Hamilton, 1992). It also includes the incubation times required to reach the desired stages.

Another advantage is that the embryo develops rapidly. Within 2-3 day of incubation, it undergoes gastrulation, neurulation and develops into a three dimensional animal with a beating heart, somites and a complex nervous system. At these early stages, the embryo is of a sufficient size to make micromanipulation practical. One such manipulation is in ovo electroporation, a technique that allows genes to be introduced and expressed in cells and tissue of the chick embryo in a spatially and temporally restricted manner. As DNA is negatively charged, an electric current can be passed across electrodes, which opens up the cell wall and depending on the position of the electrode the transfection can be targeted to a specific region. The timing of the onset of gene expression starts approximately two hours after electroporation and peaks at twenty hours after electroporation. In ovo electroporation was initially applied to transgenesis of the neural tube but it can also be used to target cells of the otic cup at E2 before closure of the otic vesicle (Momose et al., 1999; Muramatsu et al., 1997; Nakamura and Funahashi, 2001).

Classic grafting and rotation techniques have also been used to investigate the early induction and patterning of the chicken inner ear. For example Swanson et al. (1990) used grafting of a quail otocyst to the wing bud of a chicken embryo to show that formation of the inner ear epithelia can occur autonomously from early developmental stages. Fekete et al. (1998) provided evidence that hair cell and supporting cells derive from a common precursor using retroviral infection of the otic vesicle and clonal analysis.

The major disadvantage of the chicken embryo as a model system is the difficulty to obtain transgenic animals and inactivate gene function.

However recent progress in technology relying on RNA interference may facilitate loss-of-function studies in the future. On the other hand, the chicken embryo is an excellent system for gain-of-function studies. The recent introduction of the Tol2 transposon system for gene transfer in chicken is also a very significant technological advance for this type of study (see section 4.2).

## **1.3 Inner ear development**

### **1.3.1 *Otic placode induction***

The first morphological event in inner ear development in all vertebrates is the formation of the embryonic otic placode (Torres and Giraldez, 1998). The otic placode is a thickening of the surface of the head ectoderm adjacent to the hindbrain at the level of rhombomeres 5 and 6 (Noramly and Grainger, 2002). It becomes visible at 1.5 days of incubation (embryonic day (E)1.5) or Hamburger and Hamilton stage (HH) 10 in the chicken embryo. In the mouse, it is visible from E8.

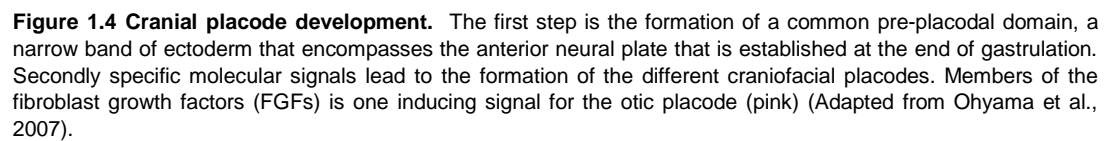
It is thought that inductive signals from the surrounding tissues (mesodermal followed by neural) lead to otic specification in the ectoderm (Schlosser and Ahrens, 2004; Streit, 2004). In a recent review by Ohyama et al. (2007) it was suggested that there are three steps in otic induction. First, the formation of the pre-placodal domain, second the induction of a “pre-otic field” within the pre-placodal domain which, after refinement, forms the otic placode. The third step is the refinement of the non-otic epidermis that surrounds the otic placode. The pre-placodal region is competent to generate any craniofacial sensory placode (nose, lens, ear, trigeminal and epibranchial ganglia and lateral line) (Reviewed in Baker and Bronner-Fraser (2001)) and can be identified by the expression of a number of genes that belong to the *Dlx*, *Six*, *Eya*, *Iro*, *BMP*, *Foxi* and *Msx* families (Brown et al., 2005; Glavic et al., 2004; Ohyama and Groves, 2004; Streit, 2004) (see figure 1.4).

*Foxi1* is one of the first otic molecular markers in zebrafish (Solomon et al., 2003). *Pax8* is the second earliest known otic marker in fish, frogs and mice, followed by *Pax2* which is initially expressed in a larger region than the otic placode in chicken and mouse embryos (Groves and Bronner-Fraser 2000). Other signalling molecules and transcription factors are expressed in the

otic ectoderm including *fgf3*, *fgf8*, *dlx3b*, *dlx4b*, *Bmp7*, *Eya1*, *GATA3*, *Nkx5.1*, *Gbx2*, *Sox3* and *Sox9a* (Reviewed in Ohyama et al. (2007)).

Fibroblast growth factors (FGFs) have been shown to be necessary for otic induction in fish, chick and mouse. FGFs are expressed in the developing hindbrain, adjacent to the presumptive otic tissue, as well as in the underlying mesoderm, however the identity and source of inducing FGFs vary from species to species. Some evidence suggests that FGF signalling is necessary and sufficient to induce early otic markers such as Pax2. Treatment with an FGF receptor inhibitor, SU5402, results in an absence of the otic vesicle and early ear markers, Pax2 and *dlx3b* in zebrafish (Leger and Brand, 2002; Maroon et al., 2002). Another pathway involved in otic placode induction is the Wnt signalling pathway. Ladher et al., (2000) first showed that Wnt8c, in combination with FGF19, augmented Pax2 expression. It has been shown that Wnt signalling mediates the placode-epidermis fate decision within the pre-otic field (Ohyama et al., 2006) and that this process requires FGF signalling. Notch signalling has been proposed to be important in the refinement of the border between the otic placode and the epidermis by regulating  $\beta$ -catenin (reviewed in Ohyama et al., 2007). There is evidence showing that elements of the Notch pathway are positively regulated by Wnt signalling and Notch1 signalling can in turn modulate the canonical Wnt signalling pathway during otic placode development (Jayasena et al., 2008).





**Figure 1.4 Cranial placode development.** The first step is the formation of a common pre-placodal domain, a narrow band of ectoderm that encompasses the anterior neural plate that is established at the end of gastrulation. Secondly specific molecular signals lead to the formation of the different craniofacial placodes. Members of the fibroblast growth factors (FGFs) is one inducing signal for the otic placode (pink) (Adapted from Ohyama et al., 2007).

### **1.3.2 From the otic placode to the inner ear: patterning and morphogenesis**

Cells from the otic placode interact with and incorporate tissue from several other embryonic sources to form the otocyst or otic vesicle. The otic placode first invaginates into the underlying mesenchyme to form the otic cup or pit, which in turn pinches off from the ectoderm to form a closed otic vesicle, a differentiated structure with sharply defined borders (Alvarez and Navascues, 1990; Bancroft and Bellairs, 1977; Torres and Giraldez, 1998). The otocyst is a hollow, pear-shaped structure that forms after neural tube closure. Following a complex series of morphogenetic events, the otocyst is transformed into the inner ear labyrinth (see figure 1.5).

The majority of the cell types found within the membranous labyrinth of the inner ear are derived from the multipotent epithelial progenitor cells that make up the otic placode. They have the potential to become any of the different cell types of the membranous labyrinth and cochleo-vestibular ganglion (CVG). The only exceptions are the Schwann cells of the CVG and the melanocytes of the secretory epithelium which are derived from neural crest cells (D'Amico-Martel and Noden, 1983; Rubel and Fritzsch, 2002). The cells of the otocyst give rise to three major cell lineages: proneural (cells that will become primary sensory neurons of the cochleovestibular ganglion which contributes to cranial nerve VIII), prosensory (cells that will develop into either sensory hair cells or supporting cells) and nonsensory. Interactions between these different lineages are also important for normal inner ear morphogenesis.

#### ***The compartment-boundary model of inner ear formation.***

A precise sequence of patterning events and cell-fate decisions, tightly regulated in space and time, is thought to create the different compartments and cell types found in the mature ear (Fekete and Wu, 2002). According to the compartment-boundary model proposed by Fekete and Wu (2002), the main axis and compartments of the inner ear are set up at the otocyst stage, under the influence of extrinsic as well as intrinsic cues.

Three main boundaries are suggested: anterior-posterior (A-P), medial-lateral (M-L) and dorsal-ventral (D-V). Altogether, these boundaries are thought to divide the otocyst into eight compartments. Many genes are expressed in a very restricted pattern in the early otocyst, but this does not prove the existence of compartments. Stronger support for the model comes

from fate map studies that have shown that the endolymphatic duct arises near the intersection of the A-P and the M-L boundaries at the dorsal pole of the chicken otocyst (Brigande et al., 2000a)(Figure 1.6).

Some of the molecular signals that pattern the otocyst along its different axes have been characterised. Transplantation experiments in chicks have shown that the A-P axis is established before the D-V axis (Wu et al., 1998). The A-P axis reflects the two domains of gene expression referred to as the anterior neurogenic domain (also known as the proneural domain) and the posterior non-neural domain. The neurogenic domain projects ventrally and the non-neural domain extends dorsally during invagination of the otic vesicle (Abello and Alsina, 2007; Abello et al., 2007). The anterior domain is characterised by the expression of several genes including Sox3, Fgf10, Lunatic fringe (Lfng), BEN, Delta1, Neurogenin-1 (Ngn1) and NeuroD. The non-neural domain expresses Irx1, Lmx1b, HNK-1, and Hairy1 (Abello and Alsina, 2007; Alsina et al., 2004; Cole et al., 2000; Goodyear et al., 2001; Myat et al., 1996; Torres and Giraldez, 1998; Vazquez-Echeverria et al., 2008). Notch signalling is involved in the establishment of the A-P patterning. Components of Notch signalling are differentially expressed between anterior and posterior regions of the otocyst. It has been shown that Notch activation is required for the restriction of the posterior genes Lmx1 and Irx1 to the non-neural domain (Abello et al., 2007). In mouse, Tbx1 is expressed in the posterior domain. Its function is thought to be to suppress or restrict anterior, neuro-sensory fate and a key downstream target of extrinsic A-P signalling (Arnold et al., 2006; Raft et al., 2004; Vitelli et al., 2003). Retinoic acid has recently been shown to be an essential determinant of A-P patterning in the inner ear (Bok et al., 2011).

The mature inner ear is also separated into a dorsal vestibular region and a ventral auditory region. The D-V axial patterning of the inner ear is established by molecules secreted by adjacent tissues; Wnts from the dorsal neural tube and Sonic hedgehog (Shh) from the neural tube floorplate and the notochord (Bok et al., 2005; Liu et al., 2002; Riccomagno et al., 2002; Riccomagno et al., 2005). The genes that are associated with the dorsal vestibular structures include; Dlx5, Dlx6, Hmx2, Hmx3 and Gbx2 and those associated with the ventral auditory and neurosensory regions include; Lfng, Ngn1, NeuroD1, Sox2 and Six1 (Fekete and Wu 2002). An experiment, whereby a segment of the hindbrain adjacent to the otocyst was rotated along its DV axis, demonstrated that genes normally expressed in the ventral region were being expressed in the dorsal region. These results

indicate that DV axis specification is mainly dependent on signals from the hindbrain (Bok et al., 2005).

The M-L axis is established last, after closure of the otic cup; though the exact timing of its specification is not clear. The endolymphatic duct is thought to arise from the medial domain and the lateral semicircular canal and ampulla are thought to arise from the lateral domain, however the anterior and posterior semicircular canals and the cochlear duct may develop from a combination of the two domains (Fekete and Wu, 2002). The genes that have been associated with the medial domain are *Gbx2*, *Pax2* (Groves and Bronner-Fraser, 2000) and the FGF receptor, *Fgfr2* (Mansour et al., 1993).

Despite these advances, it is still unclear how the different domains of the inner ear are specified or become refined from a larger domain, and how their boundaries are formed and maintained throughout development.

**Figure 1.5 Chick inner ear development.** Most of the inner ear is derived from a thickening in the ectodermal layer called the otic placode. The otic placode invaginates to form the otic cup at E2.5 which in turn pinches off to form the otic vesicle. The mature chick inner ear consists the basilar papilla (the auditory sensory epithelium, equivalent to the mammalian organ of Corti), three cristae, a utricle, a saccule, the lagena which is found at the distal end of the cochlear duct and the neglecta (not shown) (Adapted from Daudet et al., 2005).

**Figure 1.6 A compartment-boundary model of ear morphogenesis.** (a) Model of the compartmentalized otocyst viewed from anteromedial perspective, shown bisected by three boundaries (A–P, M–L and D–V) into eight developmental compartments (posterodorsolateral and posteroventrolateral not visible). (b) Several genes expressed in different parts of the otocyst are indicated, along with the compartments that they are most likely to encompass. (c) Predicted fate map for the early labyrinth (d) Possible arrangement of sensory organs and regions of the ear arising from the different developmental compartments (Adapted from Fekete and Wu 2002).  
**AC: anterior crista; BP: basilar papilla; LC: lateral crista; oC: organ of Corti; PC: posterior crista; SM: saccular macula; UM: utricular macula.**

## 1.4 Cell fate specification during inner ear development

### 1.4.1 Otic Neurogenesis

Neurogenesis in the ear begins at the placodal stage of development. First, neuroblasts are singled-out then migrate out of the anterior-ventral region (proneural domain) of the otic vesicle into the adjacent mesoderm, in a process called delamination, to form the CVG (reviewed in Alsina et al., 2003; D'Amico-Martel and Noden, 1983). The delaminated neuroblasts continue to proliferate and eventually differentiate into vestibular and cochlear neurons that will innervate the sensory organs (Alsina et al., 2004). Neural precursors express proneural genes such as *Neurogenin-1* (*Ngn1*) (Ma et al., 1998; Alsina et al., 2004). Null mutations have shown that *Ngn1* is essential for the formation of proximal, neural-crest-derived cranial sensory neurones and inner ear sensory neurones (Ma et al., 1998). *Ngn1* is also essential for expression of a cascade of basic-helix-loop-helix (bHLH) proteins.

NeuroD and NeuroM are basic helix-loop-helix (bHLH) proteins involved in neural fate determination and neuronal differentiation and survival (Ma et al., 1998, Liu et al., 2000, Kim et al., 2001) and are upregulated in epithelial neuroblasts. Mice which lack *neurogenin-1* (Ma et al., 2000) and NeuroD (Kim et al., 2001; Liu et al., 2000) do not form a normal CVG. Markers of delaminated neuroblasts include *Islet1* and  $\beta$ III-tubulin (*Tuj1*) (Li et al., 2004). Notch-Delta signalling has a crucial role in neuroblast selection in the inner ear through lateral inhibition, in a very similar way to its function during central neurogenesis and hair cell fate decisions (see later section on Notch signalling).

### 1.4.2 Prosensory patch specification

Sensory fate specification begins after neurogenesis in the avian and mammalian inner ear. The prosensory anlage (or anlagen) develops from the anterior and posterior regions of the otocyst. However it is not clear whether all of the prosensory regions are derived from a single posterior-ventral anlage that divides into two or if the two separate anlagen are specified independently. There are a number of different markers whose expression is found in the prosensory anlage, including bone morphogenetic protein 4 (*Bmp4*) (Morsli et al., 1998; Oh et al., 1996; Pujades et al., 2006; Wu and Oh, 1996), lunatic fringe (*Lfng*), jagged/serrate 1 (*Jag1*) (Cole et al., 2000), *Islet 1* (Li et al., 2004), prospero-related homeobox 1 (*Prox1*) and, FGF16

(Chapman et al., 2006). Expression of the genes, which are thought to mark prosensory specification, starts at early otocyst stages (E2 in chick and E8 in mouse) and precedes hair cell formation by several days.

Sox2, a transcription factor belonging to the group B1 Sox (SRY-related high mobility group (HMG) box) family of proteins, has recently been demonstrated to play a critical role in prosensory specification. Sox2 is widely expressed in the prosensory and proneural regions of the otocyst (Kiernan et al., 2005b; Uchikawa et al., 1999; Wood and Episkopou, 1999). Its expression overlaps with Jagged1 in putative prosensory domains and p27kip1, an established marker of the prosensory domain. Two allelic mouse mutants of Sox2, *light coat and circling (Lcc)* and *yellow submarine (Ysb)* were found to have hearing and balance impairment (Dong et al., 2002). For both of the mutants, the inner ears were shown to have abnormal morphology using the paint-filling technique. *Lcc/Lcc* mice exhibited the most severe phenotype; all three ampullae were missing, the cochlea was undercoiled and the saccule and utricle were small. The *Ysb/Ysb* mutant mice were less severely affected; the anterior and lateral ampullae were missing, the cochlea was slightly undercoiled. The *Lcc/Lcc* mice fail to differentiate hair cells and supporting cells whereas the *Ysb/Ysb* mice had some sensory formation but hair cell patterning was abnormal. These results support the role of Sox2 in prosensory specification. (Kiernan et al., 2005). There is some evidence to suggest that Sox2 acts downstream of Jagged1. Firstly, Sox2 is downregulated in Jagged1 deficient mice (Kiernan et al., 2006) and secondly there is ectopic Sox2 expression when Notch activity is artificially induced by NICD (Dabdoub et al., 2008). This data suggest that early Jagged1-mediated Notch activity induces prosensory identity through Sox2 induction.

*Eya1 (Eyes absent homolog 1)* is a transcriptional co-activator, which may also play a role in prosensory specification. *Eya1* is first expressed in the otic placode before invagination then its expression becomes restricted to the ventral region of the otocyst, from which sensory organs derive and ultimately gets restricted to hair cells. Mice deficient in *Eya1* have profound defects in inner ear development. Prosensory markers such as *Jagged1* and *Lfng* are absent in *Eya1* mice mutants and expression of sensory markers such as *Bmp4*, *Fgf3* and *Fgf10* are also absent (Zou et al., 2008). *Eya1* and Sox2 expression patterns are almost identical between E8.5 and E9.5 (Zou et al., 2008). Sox2 expression is reduced, but not completely lost, in the absence of *Eya1*. This data suggests that *Eya1* is required for sensory patch



specification and may act in parallel with Sox2. It has also been suggested to act after prosensory specification as its expression is downregulated in Jagged1 conditional mutants (Kiernan et al., 2005b; Kiernan et al., 2006).

The factors that regulate the formation of the sensory epithelium from a population of otic progenitor cells remain unknown; however recent advances in this field have provided valuable insights regarding the role of the Notch signalling pathway during inner ear development. This thesis will in part be exploring these functions further and provide new insights into possible roles during chick inner ear development.

### **1.4.3 *Specification of hair cells and supporting cells***

After the prosensory domains have been specified, individual cells within these domains have to decide whether to develop as a hair cell or support cell. Lineage studies have confirmed that sensory hair cells and supporting cells arise from common progenitors (Fekete et al., 1998). In the chick, retroviral-mediated lineage analysis has been used to investigate clonal relationships and dispersion of epithelial, neuronal and mesenchymal cells during inner ear development (Lang and Fekete, 2001). The alternating arrangement of hair cells and supporting cells is regulated by lateral inhibition mediated by Delta/Notch signalling. Previous studies have shown that the primary fate choice within the population of prosensory cells is a hair cell fate (Brooker et al., 2006; Kiernan et al., 2006; Kiernan et al., 2005). The basic-helix-loop-helix transcription factor, Atoh1, is necessary and sufficient to induce a hair cell fate (Gubbels et al., 2008; Kawamoto et al., 2003; Woods et al., 2004; Zheng and Gao, 2000).

## 1.5 Notch signalling

The Notch signalling pathway is an evolutionary conserved cell-cell communication pathway present in all Metazoans. It is most famous for its role in lateral inhibition that regulates cell differentiation and proliferation in a wide variety of embryonic and adult tissues, such as the gut, brain, blood vessels and the inner ear (reviewed in Artavanis-Tsakonas et al., 1999; Lewis, 1998).

### 1.5.1 The Notch receptor

The Notch gene was identified over 90 years ago in a strain of *Drosophila melanogaster* displaying notches at the wing margin (Mohr, 1919). It was later shown that the Notch gene encodes a 300kDa single-pass transmembrane receptor (Kidd et al., 1989; Kidd et al., 1986; Wharton et al., 1985). In *Drosophila* there is only one Notch-encoding gene. In mammals, four receptors have been identified so far (Notch 1-4). The Notch receptors are type-I transmembrane heterodimers that consist of a conserved extracellular domain of up to 36 epidermal growth factors (EGF)-like repeats, involved in ligand interactions, and three juxtamembrane Lin-12-Notch repeats (LNR), involved in modulating interactions between the extracellular and the membrane-tethered intracellular domains (Kidd et al., 1989; Kidd et al., 1986; Wharton et al., 1985). The transmembrane-intracellular domain contains a RAM (RBP-jk-associated molecule) domain, followed by seven highly conserved ankyrin (ANK) repeats. Nuclear localisation signal sequences and a proline, glutamine, serine, threonine-rich (PEST) domain regulating protein stability are found C-terminal to the ANK domain. Notch-1 and -2, but not Notch-3 and -4, contain a transactivation domain (TAD) located C-terminal to the ANK repeats (Reviewed in Lubman et al. (2004).

Following the identification of the Notch gene, loss of function experiments caused a “neurogenic” phenotype, where cells that were destined to become epidermis switch fate and give rise to neural tissue reviewed in (Artavanis-Tsakonas et al., 1999; Fiuza and Arias, 2007). However Notch is pleiotropic, thereby affecting many tissues. Mutations of Notch receptors or ligands cause several diseases in humans such as Alagille syndrome, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), T-cell leukemia, aortic valve calcification and cancer.

### **1.5.2 The canonical Notch signalling pathway**

The Notch signalling pathway is a cell-cell communication pathway which does not involve a second messenger. Activation of Notch receptors occurs upon binding to membrane-bound ligands of the Delta, Serrate (also named Jagged in mammals) and Lag-2 (DSL) families. In mammals, there are five genes encoding transmembrane ligands, Jagged1 (Jag1), Jagged2 (Jag2), Delta-like1, Delta-like3 and Delta-like4, identified so far (reviewed in D'Souza et al., 2010). Notch ligands are also type-I transmembrane proteins. Following ligand binding, the Notch receptor undergoes a series of proteolytic cleavages in the “signal-receiving” cell (Kopan and Turner, 1996) (Figure 1.7). Notch is first cleaved by the ADAM-family of metalloproteases, ADAM10/TACE (TNF- $\alpha$ -converting enzyme), at the extracellular domain (S2 cleavage). The Notch intracellular domain (NICD) is then cleaved by the  $\gamma$ -secretase complex (S3 cleavage), which consists of presenilin, nicastrin, PEN2 and APh1 (De Strooper et al., 1999; Mumm et al., 2000). The second cleavage releases NICD which then translocates to the nucleus and acts as a transcriptional co-activator. NICD cannot bind directly to DNA, but heterodimerizes with transcription factors of the CSL (CBF1 in mammals, Suppressor-of-Hairless [Su(H)] in *Drosophila* and Lag-1 in *Caenorhabditis elegans*) family also called RBP-Jk (recombination signal sequence-binding protein Jk). In the absence of NICD, CSL proteins complex with ubiquitous co-repressor proteins, such as SMRT (silencing mediator of retinoic and thyroid) hormone receptors, to repress transcription of Notch target genes. The NICD generated upon ligand binding competes with the repressor proteins and forms a NICD-CSL complex. This complex binds with Mastermind (MAM) protein and recruits the ARC-L/MED mediator complex (Fryer et al., 2004), the histone ubiquitin ligase, Bre-1 (Bray et al., 2005) and histone acetyltransferases to assemble an active transcription complex on target promoters.

**Figure 1.7 The Notch signalling pathway and target gene activation.** The Notch receptor is activated upon binding of the Notch ligand. First, an ADAM metalloprotease catalyzes a specific cleavage (S2) within the Notch extracellular domain. Subsequently  $\gamma$ -secretase catalyzes the cleavage of the Notch intracellular domain (NICD) (S3). NICD migrates into the nucleus of the cell and interacts with RBP-J and recruits a co-activator complex composed of mastermind (MAML-1) and other chromatin modifying transcription factors resulting in transcriptional activation of Notch target genes. From Borggreffe and Oswald (2009).

### 1.5.3 *Notch target genes*

At present there are a limited number of Notch target genes that have been identified. The genes of the *Hairy*-and *Enhancer-of-Split* in *Drosophila* and the related Hes and Hey genes in mammals encode highly conserved basic helix-loop-helix (bHLH) proteins. These genes are the most extensively studied primary targets and effectors of the Notch signalling pathway. In *Drosophila* they control developmental processes such as segmentation, myogenesis and neurogenesis (Fischer, 2007; Iso et al., 2003). The bHLH proteins consist of the DNA-binding basic domain (b), followed by two amphipathic  $\alpha$ -helices separated by a loop (HLH), followed by the Orange domain (O) which consists of two  $\alpha$ -helices. The Hes proteins act as transcriptional repressors that regulate gene expression in cells in which Notch is active, affecting processes such as cell differentiation and proliferation. The O domain acts as a transcriptional repressor when fused to the DNA-binding domain. Other characteristics of the Hes proteins include an invariant proline residue in the basic domain and a highly conserved carboxyterminal tetrapeptide motif WRPW. In mammals seven *Hes* genes (*Hes 1-7*) and three *Hey* genes (*Hey 1, 2, L*, also referred to as *Hrt1,2,3*; *Her1,2*; *Herp2,1* or *Chf2,1*), a subfamily of Hes, related with a YRPW motif, have been identified. Hes proteins bind to N- and E-box DNA sequences (CACNAG, CANNTG) and can recruit corepressors through their WRPW tetrapeptide (Iso et al., 2003). Hey proteins however have an invariant glycine residue in their basic domain and they do not bind to N-box sequences.

Loss-of-function of these genes, in *Drosophila*, leads to an increase in neuroblasts at the expense of epidermal precursors reviewed in Artavanis-Tsakonas et al. (1999). There several studies which have shown that most of these genes are direct Notch targets, whereas Hes2, Hes3 and Hes6 appear to be independent of Notch signalling. (Reviewed in Borggreffe and Oswald (2009). A constitutively active form of Notch1 has been notably shown to activate the promoters of *Hes1*, *Hes5*, *Hes7* and related genes *Hey1*, *Hey2* and *HeyL* (reviewed in Borggreffe and Oswald, 2009).

## 1.6 Notch signalling during inner ear development

Through its effects on gene expression, Notch signalling creates differences between signal-sending cells (expressing a Notch ligand) and signal-receiving cells (in which Notch receptors are activated). However the cellular outcomes of Notch activation are very diverse, depending on the

cellular context and the particular tissue considered. Another source of variability stems from the fact that the expression of Notch ligands is itself regulated by Notch activity. Depending on the mode of regulation, negative or positive, interacting cells will “compete” for either high or low levels of Notch activity, or conversely “cooperate” for maintaining high levels of Notch activity. These contrasting modes of Notch signalling have been respectively termed lateral inhibition and lateral induction (Figure 1.8).

### **1.6.1    *The mechanism of lateral inhibition***

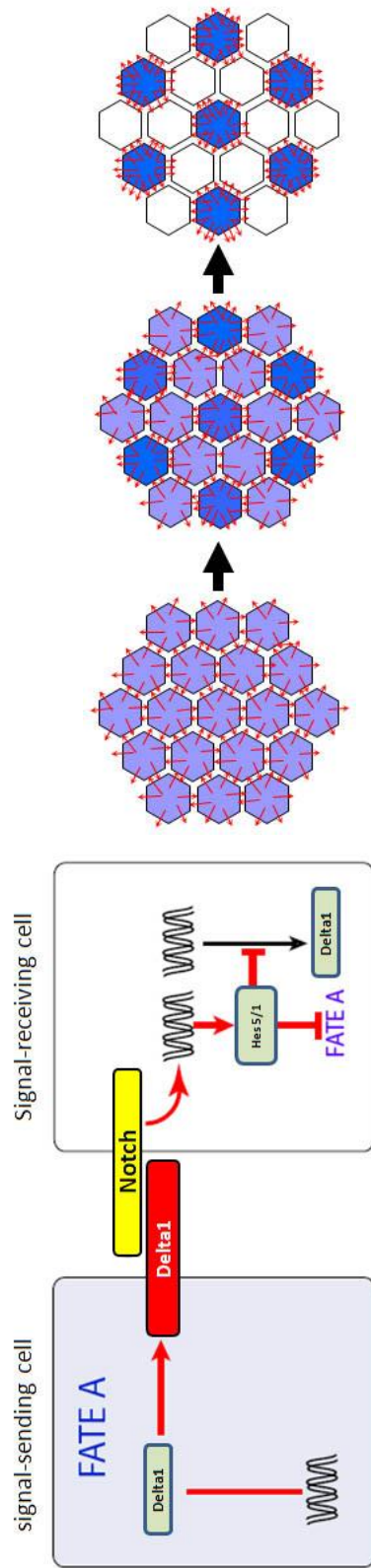
Notch signalling is most famous for its role in lateral inhibition whereby signal-sending cells, which adopt a “primary” fate and express a Notch ligand, prevent neighbouring cells from doing likewise. In the signal-receiving cells, Notch activity inhibits adoption of the primary fate and represses the expression of Notch ligands. This negative feed-back loop amplifies differences in expression of Notch ligands between initially equivalent cells, and generates “salt-and-pepper” patterns of cell differentiation. This mechanism, originally described during the determination of neuronal precursors in the *Drosophila* nervous system (Heitzler and Simpson, 1991), is crucial for the development and maintenance of several vertebrate tissues such as the CNS (Chitnis, 1995; Henrique et al., 1995), the gut (Lewis, 1998; Sancho et al., 2004) and the hematopoietic system. In the embryonic CNS of vertebrates, lateral inhibition controls neuronal differentiation and ensures that a pool of cycling progenitor is maintained throughout neurogenesis. The nascent neurons express the ligand Delta and by activating Notch receptors in neighbouring cells, they prevent these from becoming neurons themselves. When Notch activity is disrupted, excess neurons are formed prematurely, and the pool of progenitors is depleted. This is exemplified in a classic neurogenic mutant, the zebrafish *mindbomb* (Haddon et al., 1998; Jiang et al., 1996), in which the processing and function of Notch ligands is altered.

### **1.6.2    *The mechanism of lateral induction***

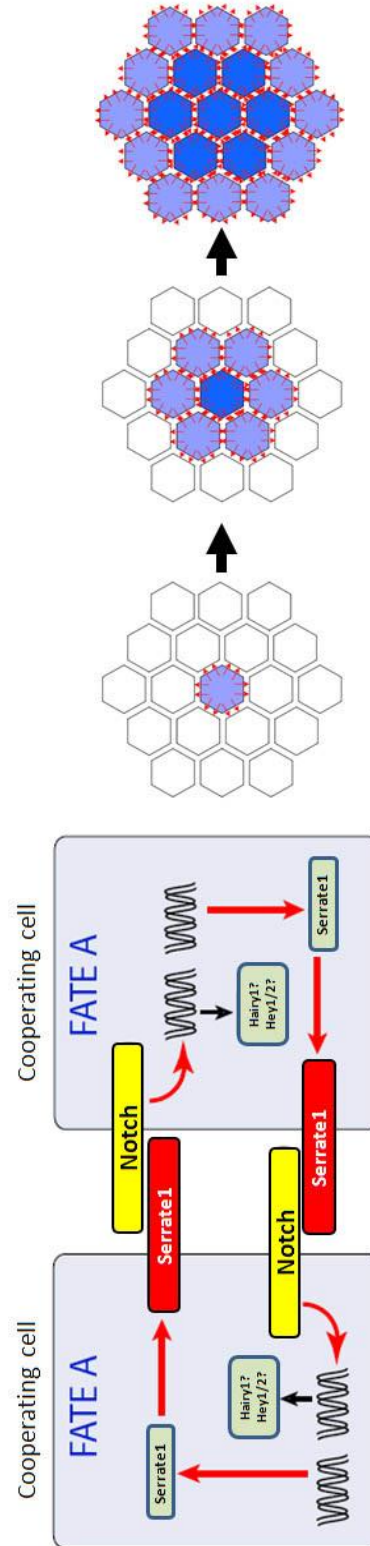
Conversely, Notch activity can promote the expression of Notch ligands, a process known as lateral induction. In this case, if a cell expresses a raised level of ligand it will signal to its neighbours to do the same. Thus the interacting cells cooperate to maintain high levels of Notch ligands and jointly adopt a particular route of differentiation. This all-or-none behaviour prevents the pepper-and-salt pattern that occurs with lateral inhibition and promotes the formation of sharply defined boundaries of gene expression. There are fewer examples of lateral induction than of lateral inhibition

described so far, the most famous being the specification of the dorso-ventral (d/v) boundary cells of the wing imaginal disc in *Drosophila*, where restricted activation of Notch over a prolonged period is required for the growth and the patterning of the wing-margin (de Celis et al., 1996). The d/v boundary forms at the interface between two cell populations, dorsal and ventral, which express two different Notch ligands. Serrate is expressed in dorsal cells and activates Notch in ventral cells and Delta1 is primarily required in ventral cells to signal to the dorsal cells. Experiments have shown that Serrate and Delta1 induce each other's expression in a dorsal-ventral asymmetric way which places them in a positive feedback loop (de Celis and Bray, 1997; Panin et al., 1997).

## Lateral Inhibition



## Lateral Induction



**Figure 1.8** In **LATERAL INHIBITION**, Notch activity prevents the expression of Notch ligands and inhibits the fate A. Differences in levels of Notch activity are amplified and interacting cells adopt different fates. In **LATERAL INDUCTION**, Notch activity promotes the expression of Notch ligands, creating a positive feedback loop. Interacting cells "cooperate" to maintain high levels of Notch activity and they adopt the same fate A.



### ***1.6.3 Notch-mediated lateral inhibition and lateral induction play important roles during inner ear development***

In the developing ear, Notch signalling acts at different stages, and in different ways, to regulate neuroblasts production, hair cell and supporting cell fate decisions, and the formation of the prosensory domains.

#### ***Lateral inhibition dependent on Delta1 and Jagged2 regulates neuroblast and hair cell formation***

The neuroblasts that give rise to auditory and vestibular neurons delaminate from the anterior region of the otic cup/vesicle (HH11). Their formation is controlled by Notch-mediated lateral inhibition, in a process very similar to neurogenesis in the CNS. The neuroblasts express the bHLH proneural transcription factor Ngn1, which is essential for their formation (Ma et al., 2000; Ma et al., 1998), and the Notch ligand Delta1 (Morrison 1998; Adam 1998; Alsina et al., 2004), while the Notch receptor is expressed throughout the otic placode/cup. Ngn1 is required for Delta1 expression, while Notch activity inhibits Ngn1 and Delta1 expression (Ma et al., 1998); (Raft et al., 2007). Hence, when a neuroblast is formed, it activates Notch receptors in neighboring cells, preventing these from becoming neuroblasts. When Notch signalling is disrupted, the expression of Ngn1 and Delta1 is greatly upregulated and excess neuroblast production occurs (Daudet et al., 2007; Haddon et al., 1998; Ma et al., 1998). In the chick embryo, several members of the Hes5 family are expressed in the anterior region of the otic cup, suggesting that they could be acting as effectors of the Notch pathway during the lateral inhibition of neuroblast formation (Abello et al., 2007; Alsina et al., 2004; Daudet et al., 2007).

The most studied aspect of Notch signalling in the developing inner ear is its implication in the control of hair cell formation. Sensory hair cells and non-sensory support cells are arranged in a regular, alternating pattern. Such highly ordered cellular pattern suggested that lateral inhibition may be regulating the differentiation of hair cells and supporting cells (Corwin et al., 1991; Lewis, 1991). The first experimental evidence supporting this hypothesis was given by Kelley et al. (1995) who showed that the ablation of single hair cells in the embryonic organ of Corti could induce some of the surrounding supporting cells to switch fate and become hair cells. This indicated that immature hair cells normally inhibit the surrounding cells from becoming hair cells. However specific molecular signalling pathways were not identified. It was later shown that hair cells express Delta1 and Jagged2 (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999) and

defective Notch signalling results in the overproduction of hair cells. In the zebrafish *Mind bomb (mib)* mutant, lacking an ubiquitin ligase required for Delta function (Itoh et al., 2003), hair cells are produced in great excess and prematurely at the expense of supporting cells (Haddon et al., 1998). Similar phenotypes, although varying in severity, have then been reported in the inner ear of mice carrying mutations of the Delta1 (Kiernan et al., 2005a); (Brooker et al., 2006), Jagged2 (Lanford et al., 1999; Lanford et al., 2000), *Notch1* (Kiernan et al., 2005a) and *Rbpsuh* (Yamamoto et al., 2006) genes.

The main effectors of Notch during lateral inhibition of HC formation are Hes5 and Hes1. In the developing mouse cochlea Hes1 is expressed in the greater epithelial ridge (GER) and in the lesser epithelial ridge (LER) whereas Hes5 is predominantly expressed in the LER in supporting cells and in a narrow band of cells in the GER (Zine et al., 2001). Hes1<sup>-/-</sup> mice exhibited an increase in the number of IHC whereas Hes5<sup>-/-</sup> mice (Zine et al., 2001) exhibited a significant increase in the number of OHC in the cochlea and formation of supernumerary hair cells in the vestibular system, which also showed an upregulation of Math1 (Zine et al., 2001). Hes1 and Hes5 are thought to inhibit HC differentiation by antagonizing the function of the pro-hair cell transcription factor, *Math1*: in postnatal rat organ of Corti explants, Hes1 transfection prevents the ectopic formation of hair cells induced by Math1 overexpression (Zheng et al., 2000). By limiting the expression of the pro-hair cell transcription factor Atonal1/Math1, Notch signalling ensures the harmonious production of hair cells and supporting cells, which is crucial for proper inner ear function.

#### *Jagged1 regulates the formation of prosensory domains through lateral induction*

The Notch ligand Jagged1 (Serrate1 in the chick) is not expressed by hair cells, but within supporting cells and progenitor cells of the immature sensory patches. Early studies in ENU-mutated mice have shown that loss of Jagged1 function is associated to a loss of sensory crista and a reduction in the number of hair cells in the organ of Corti (Kiernan et al., 2001; Tsai et al., 2001). Later experiments using conditional alleles of Jagged1 confirmed these observations and suggested that Jagged1 has a specific function in the formation of the sensory patches (Brooker et al., 2006; Kiernan et al., 2001). These results suggested an early role for Notch signalling via Jagged1 in prosensory specification.

In the chicken embryo, *Serrate1* is expressed in the regions of the otocyst which are known to give rise to the sensory domains: the anterior neurogenic patch, and the posterior domain (non-neural region) of the otic cup (HH11) (Abello and Alsina, 2007; Adam et al., 1998; Cole et al., 2000; Myat, 1995; Myat et al., 1996). *Serrate1* expression is maintained within the differentiating sensory domains throughout inner ear development. As opposed to *Delta1*, *Serrate1* expression is positively regulated by Notch activity (Daudet and Lewis, 2005; Eddison et al., 2000); and it has been proposed that lateral induction dependent on *Serrate1* could be involved in the expansion/formation of the prosensory patches (Daudet and Lewis, 2005). Inhibition of Notch activity, using the gamma-secretase inhibitor, DAPT, *in vitro*, leads to a loss of prosensory marker expression. Ectopic expression of constitutively active form of Notch1 (NICD) leads to the expression of prosensory markers in embryonic mammalian cochlea and in the developing chick inner ear leads to ectopic sensory patches. Deletion of other prosensory markers such as *Lfng* and *BMP4* do not lead to a loss of hair cells or supporting cells (Chang et al., 2008; Zhang et al., 2000).

## 1.7 Aim

Despite the recent advances in understanding the functions of the Notch pathway during inner ear development, some important questions remain. Sensory progenitors uniformly express high levels of Jagged1/Serrate1 at the time of hair cell production, yet some progenitors “escape” Notch activity to differentiate into hair cells. How do hair cell fate decisions occur and is this process strictly dependent on a decrease in intrinsic levels of Notch activity? If so, what are the dynamics of this process? Are there differences in the way Notch activity is regulated within proliferating progenitor cells, postmitotic uncommitted cells, or cells that form the border of the sensory domains? What is the exact role of lateral induction during sensory patch formation? It is not known whether it functions to promote expansion of prosensory patches or if it is required for the formation of boundaries between sensory and non-sensory regions.

The main aim of my project is to investigate these questions in the developing chicken inner ear, using genetically encoded, fluorescent reporters of Notch activity and secondly to test the roles of lateral induction using a novel gain-of-function approach.

Genetic reporters are useful tools for identifying where and when a gene of interest is expressed as they drive the expression of a fluorescent protein such as EGFP. In this study I tested different genetic reporters of Notch activity and identified one construct, based on cis-regulatory elements of the mouse *Hes5* promoter (Nelson et al., 2006; Ong et al., 2006), which can be used as a reporter of Notch activity in the otic cup and early otocyst. I verified that this reporter exhibit dynamic responses to variations of Notch activity using DAPT (a  $\gamma$ -secretase inhibitor) to block Notch signalling, and overexpression of the chicken Notch intracellular domain or Delta1 to over activate Notch signalling. In order to induce stable integration of this reporter within the developing inner ear, I next cloned the reporter elements into an RCAN retroviral vector (Petropoulos et al., 1992) and Tol2 transposons which enabled the analysis of reporter activity within the sensory patches at the time of hair cell production. Using immunohistochemistry I confirmed that the reporter is only active in supporting cells and sensory progenitor cells. Furthermore, time-lapse imaging of DAPT-treated sensory epithelia showed that the reporter activity is rapidly extinguished when Notch activity is blocked. Variations in levels of fluorescence were seen within individual cells over time. In order to test the role of lateral induction directly, the *Hes5* promoter was cloned

upstream of Delta1 this also provided further insights into the role of lateral inhibition.

# 2

## MATERIALS AND METHODS

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### 2.1 Animals

Fertilised White Leghorn chick (*Gallus gallus*) eggs were obtained from Henry Stewart Ltd. and incubated at 38°C and 30-80% humidity for designated times. The embryos were staged according to Hamburger-Hamilton (HH) tables (Hamburger and Hamilton, 1992). Embryos older than embryonic day (E) 5 were killed by decapitation otherwise the whole embryo was fixed in 4% Paraformaldehyde (PFA).

### 2.2 DNA manipulation and molecular cloning

#### 2.2.1 Digestion of plasmid DNA by restriction endonucleases

The restriction digest mix consisted of 5 units of enzyme per 1 µg of DNA (a five-fold excess), 1x enzyme buffer and water to make up to 50 µl. The mix was incubated at 37°C for 2 hours. The enzyme was inactivated at 65°C for 15 minutes (when appropriate-some are not heat-inactivatable).

#### 2.2.2 Agarose gel electrophoresis and DNA extraction

A 1% agarose gel was typically used. 1 g of agarose was dissolved in 100 ml of 1x Tris-acetate-EDTA (TAE) (0.04 M Tris-acetate, 0.001 M EDTA) using a microwave. The 1x TAE is made from a 50x stock TAE solution (for 500 ml):

- 121 g Tris base
- 28.6 ml glacial acetic acid
- 50 ml 0.5 M EDTA (pH 8.0)

Once the agarose was dissolved and cooled, 7 µl of ethidium bromide solution (10 mg/ml) (Electran® BDH) was added. The solution was then poured into a gel mould with combs of appropriate depth to hold the required volume.

Following a restriction digest the insert of interest was extracted from agarose gel. All of the digest mix was run on a 1% agarose gel. The band of interest was excised out using a scalpel, under UV illumination, which was kept to a minimum. The DNA was extracted from the band using Wizard sv Gel and PCR clean-up system (Promega), according to the manufacturer's instructions.

### **2.2.3 DNA ligations**

The following mixture was typically used for DNA ligations:

- 1 µl 1-3 units of T4 DNA ligase (Promega)
- 1 µl 10-30ng Linearised vector
- 3 µl 10-30ng Insert
- 5 µl 2x T4 DNA ligase buffer (Promega)

Ligations reactions were usually performed at more than one vector:insert ratio (usually 1:0, 3:1, 1:1, 1:3) and the final volume adjusted to 10 µl with water. Ligation mixes were incubated overnight at 16°C.

### **2.2.4 Transformations of competent bacteria**

DH5α competent *E. coli* cells (subcloning efficiency (used for routine amplification) and high efficiency) (New England BioLabs), were thawed on ice. Transformation tubes were also placed on ice. 50 µl of bacteria was carefully pipetted into the bottom of the tube. 5 µl of the ligation mix or 100 pg-1 µg of plasmid DNA was added to the bacteria. This was mixed by gently flicking the tube 4-5 times. The reaction was incubated on ice for 30 minutes followed by a 30 second heat shock at 42°C using a water bath. The tubes were then placed back on ice for 5 minutes. 1ml of LB or SOC was added to the reaction at room temperature. The reactions were incubated for one hour in a shaker incubator at 37°C, 230 rotations per minute. 100 µl was spread onto LB-Agar plates containing either ampicillin or kanamycin and incubated upside-down in a 37°C oven overnight. Individual colonies were picked using a 200 µl pipette tip and grown overnight in LB with an appropriate selection marker for mini preps (2 ml) or midipreps (50 ml), or used for colony PCR. Alternatively the plates were stored at 4°C until required.

### **2.2.5 Screening colonies by PCR**

A colony was picked with a pipette tip and copied onto a numbered grid on a new plate by touching the tip on the surface of the agar. The tip was then placed into a PCR tube containing 4 µl of water and incubated for 5 minutes at room temperature. The solution was pipetted up and down to mix. The

PCR tube was incubated at 95°C for 10 minutes. 6 µl of PCR master mix was added to reach 10 µl final volume.

The following mix was used for a 25 µl reaction volume :

- 1 X Go Taq® Green Master Mix (2X) 12.5 µl
- 1 µM upstream primer (10 µM) 2.5 µl
- 1 µM downstream primer (10 µM) 2.5 µl
- <250 ng DNA template 5 µl
- Nuclease-free water 2.5 µl

Following PCR the samples were run on a 1% agarose gel and visualised (see section 2.2.2).

## **2.2.6 Plasmid DNA purification and precipitation**

### ***MiniPrep***

A single bacterial colony picked from an LB-agar plate, was grown in 5ml LB with 1x ampicillin/kanamycin (100µg/ml), for 16 hours. 4 ml of bacteria culture was separated into two 2 ml eppendorf tubes. 500 µl of the remaining culture was stored in 100% glycerol (50:50) in -80°C. The bacteria culture in the 2 ml eppendorf tubes was centrifuged at 3000 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended and DNA was purified according to the instructions of the PureYield™ Plasmid Miniprep System kit (Promega).

### ***MidiPrep***

A single bacterial colony picked from an LB-agar plate, was grown in 50ml LB with ampicillin (100 µg/ml), for 16 hours at 37°C with vigorous agitation. The 50 ml culture was transferred to a 50 ml falcon tube and centrifuged at 5000 rpm for 20 minutes at 4°C. The bacterial pellet was resuspended and DNA was purified according to the instructions of the PureYield™ Plasmid Midiprep System kit (Promega).

### ***Phenol/Chloroform DNA extraction***

Plasmid DNA eluted from the Midiprep columns was further purified using phenol/chloroform extraction. The DNA solution was made up to 200 µl with water. An equal volume of Tris-buffered phenol:chloroform:isoamylalcohol (25:24:1) (Sigma-aldrich®) was added to the DNA solution. The mixture was vortexed for 1 minute, then spun at full speed in a microcentrifuge for 5 minutes. The upper, aqueous phase was transferred to a fresh tube, being



careful to avoid the interface. In order to remove traces of phenol, an equal volume of chloroform:isoamyl alcohol (24:1) was added to the aqueous layer. The mixture was again vortexed for 1 minute, then spun at full speed in a microcentrifuge for 5 minutes. The upper, aqueous phase was transferred to a fresh tube. The DNA solution was then ready to be ethanol precipitated.

#### ***Plasmid DNA precipitation and resuspension***

Precipitation of the plasmid DNA was performed either after phenol chloroform extraction or straight from the eluted DNA. A 0.1 volume of 3M sodium acetate and 2.5 volumes of 100% Ethanol (kept at -20°C) was added to the DNA solution, then vortexed and incubated at -20°C for one hour. Following incubation the solution was centrifuged at 15000 rpm for 10 minute at 4°C. The liquid was discarded and the pellet of DNA was then washed in 70% ethanol then centrifuged for a further 5 minutes. The pellet was left to air dry (taking care not to dry out the pellet too much). The DNA pellet was then resuspended in water (typically 30-50 µl, depending on the size of the pellet).

#### **2.2.7 Measuring the concentration of plasmid DNA**

The concentration of the DNA was determined by measuring the absorbance of the DNA at 260 nm using a spectrophotometer. The value obtained was converted to a concentration using the following equation:

$$\text{DNA concentration } (\mu\text{g}/\mu\text{l}) = \frac{(\text{absorbance } 260\text{nm}) \times \text{Dilution factor} \times 50}{1000}$$

#### **2.2.8 Sequencing Plasmid DNA**

The following mix was used to sequence DNA:

- 400ng template DNA
- 1 µl of 3.2 µM primer
- Water to 6 µl
- 4 µl Big Dye Terminator mix (Perkin Elmer)

The above mix was used in PCR with the following thermal cycler programme:

1. 96°C for 3 minutes
2. 96°C for 30 seconds
3. 50°C for 15 seconds
4. 60°C for 4 minutes
5. Repeat steps 2 to 4 for 25 cycles
6. 4°C hold

### *Precipitation*

10 µl of water was added to bring the volume up to 20 µl. The PCR reaction was transferred to a 1.5 ml microfuge tube and precipitated as in 2.2.6. The DNA pellet was air-dried and sent to the UCL sequencing service.

### **2.2.9 Plasmids and molecular cloning**

The pNICD expression construct encoding a flag-tagged full-length chicken Notch1 intracellular domain was kindly provided by Dr Y. Wakamatsu (Wakamatsu et al., 1999). Replication-competent avian retrovirus (RCAS) encoding the full length chicken Delta1 (RCAS(B)-Delta1; (Henrique et al., 1997) was also used. A plasmid driving the expression of a red fluorescent protein under the control of a CMV promoter (pDsRed; pDSRED2-C1, Clontech) was co-electroporated in some experiments to visualise transfected cells.

Two different types of genetic reporters were tested: **pCBF-d2VenusYFP** (Gift from Dr C Rallis, Cancer Research UK), containing 5 tandem repeats of CSL binding motifs (CBS) upstream of a minimal SV40 promoter and the destabilised Venus Yellow Fluorescent Protein (d2VenusYFP) coding sequence. **pHes5-d2EGFP** (Gift from Prof R. Kageyama, Kyoto University, Japan; see (Takebayashi et al., 1995) that consisted of 700bp of the mouse Hes5 promoter region upstream of destabilised EGFP coding sequence. This construct was previously used by Nelson et al. (2006) to monitor Notch activity in chick retinal progenitor cells.

#### ***pRCAN-Hes5-d2EGFP***

The pRCAN-Hes5-d2EGFP construct was produced as follows: The forward primer 5'-CACGTAT**CG**ATTTACTAGCGCTACCGGACTCA-3' and the reverse primer 5'-GGCGCAT**CG**ATTCTACACATTGATCCTAGCAG-3' were used to amplify Hes5-d2EGFP (1.6kb) from the pHes5-d2EGFP plasmid using the high fidelity Taq *pfu* polymerase (Promega). The primers were designed to contain Cla1 restriction sites (in **bold**). The PCR product was digested with Cla1. The insert was ligated into the Cla1 site of the linearised and dephosphorylated vector, RCANBP(B) (a kind gift from Dr. Stephen Hugues), hereafter referred to as RCAN. Two RCAN-Hes5-d2EGFP clones with the Hes5-d2EGFP cassette in either the same or the opposite orientation relative to the *gag-pol-env* coding sequences of RCAN were sequenced and selected for further work.

### ***RCAS-EGFP***

pSlax-GFP (cloned by N. Daudet) was digested with Cla1 to excise the EGFP coding sequence (800bp). The insert was ligated into the Cla1 site of the RCASBP(B) vector (a kind gift from Dr Stephen Hughes), hereafter referred to as RCAS. Colonies were screened by PCR for correct orientation using two sets of primers: RCAS forward with GFP reverse and RCAS forward with RCAS reverse.

### ***pT2K-CAGGS (empty)(6.2kb)***

The empty pT2K-CAGGS vector was generated by N. Daudet by digesting the pT2K-CAGGS Tol2 plasmid (a gift of Dr Yoshiko Takahashi, Nara Institute of Science and Technology, Japan) with Sal1 and Xho1 to excise the CMVIE and beta-actin promoter. The sticky-ends were re-ligated to produce the final vector.

### ***pT2K-Hes5-d2EGFP (7.8kb)***

The pTol2-Hes5-d2EGFP was produced by excising Hes5-d2EGFP cassette from pRCAN-Hes5-d2EGFP with Cla1. The ends of the insert were blunted by incubation with the DNA polymerase large (Klenow) fragment (Promega) according to the manufacturer's instructions. Following Klenow inactivation or purification, the insert was ligated into the empty pT2K-CAGGS vector, which was linearised with EcoRV to create blunt-ends and dephosphorylated. The orientation of the insert was verified by digestion with Not1 and EcoR1. Analysis of the results confirmed that the constructs can be used in with insert in either orientation.

### ***pT2K-Hes5-Delta1-IRES-EGFP (10.6kb)***

The pT2K-Hes5-Delta1-IRES-EGFP was produced as follows: pSlax-Hes5 (cloned by N. Daudet) was digested with SacI to excise the Hes5 promoter region (760bp). The insert was ligated into the SacI site of the PCAS-Delta1-IRES-EGFP vector (a gift from Domingos Henrique). The orientation was checked by restriction digestion with SacI and sequencing: The forward primer '5-CCTAGAAGTACGCTTGGCA-3' and the reverse primer 5'-TTACTTGTACAGCTCGTCCATGCCG-3' were used to amplify Hes5-Delta1-IRES-EGFP (4.4kb) from the PCAS-Hes5-Delta1-IRES-EGFP construct using Phusion polymerase according to the manufacturer's instructions (New England Biolabs). The insert was ligated into the empty pT2K vector, which was linearised with EcoRV and dephosphorylated.

### *pT2K-TomatoNLS*

This plasmid (cloned by N. Daudet) was constructed by ligating the coding sequence of a nuclear-localised Tomato red fluorescent protein, which was excised from pCAS-nTomato, into pT2K-CAGGS.

## **2.3 Otic cup transfection by In Ovo electroporation**

### **2.3.1 Plasmids**

Plasmid DNA used for electroporation was prepared using either the Qiagen Midiprep (Qiagen, UK) or the Promega Ultrapure Midiprep (Promega, UK) systems. Plasmids were diluted at the desired final concentration (typically 1 mg/ml for each construct) with water containing 1% sucrose and Fast Green dye (Sigma) for easy visualisation of the DNA solution.

### **2.3.2 Preparation of the embryos**

For the transfection of the otic cup, eggs were incubated horizontally for 48-56 hours in order to reach stage 13-15. The eggs were removed from the incubator and disinfected with 70% Ethanol. Two small holes were made in the shell: one on the top of the egg to release pressure and one at the broad end from which 2 ml of albumen was pulled out with a syringe fitted with a gauge needle. A 2 cm diameter window was made on top of the egg with small scissors. The vitelline membrane covering the embryo was pierced using a needle and a drop of Phosphate Buffer Saline (PBS; pH 7.4) was applied at the surface of the embryo. It was then removed from the head region of the embryo to expose the otic cup.

### **2.3.3 Injection of the DNA solution into the otic cup**

The DNA solution was injected using a micropipette into the right otic cup. Micropipettes were made from glass capillary tubes 1.2mm x 0.94mm in diameter (Harvard Apparatus, Ltd). The micropipettes were filled using gel-loading tips (Eppendorf) and were mounted in a micropipette holder on a micromanipulator. To transfect the otic cup, the tip of the micropipette was positioned within the lumen of the cup, with care not pierce the tissue, and DNA was blown continuously until the cup was filled. The injection of the DNA solution was controlled by mouth.

### **2.3.4 Electroporation**

Electrodes (cathode = tungsten needle; anode = gold-coated platinum electrode) were positioned using micromanipulators on either side of the otic cup, without touching any embryonic tissue. The tungsten needle (negative)

was positioned within the otic cup lumen along with the filled micropipette so that the tips meet. The straight or L-shaped platinum electrode (positive) was positioned on the opposite side. The distance between the electrodes was approximately 4 mm. A BTX ECM 830 Electro Square Porator™ was used to generate a series of three 100-millisecond bursts of 30Hz, 7 volt square-wave electric pulses at 500ms intervals. DNA solution was blown into the otic cup simultaneously. After electroporation, a few drops of PBS were applied to wash off excess DNA solution. The eggs were sealed with Sellotape® and returned to the incubator from 24 hours up to a maximum of 8 days.

## 2.4 In situ hybridisation

### 2.4.1 DIG-labelled RNA probe synthesis

RNA probes labelled with digoxigenin (DIG) were transcribed from plasmid DNA containing partial cDNA sequences of *Hes5.1* and *Hes5.3* genes (a gift of Dr D. Henrique, Lisboa, Portugal). First the plasmids were digested with a Not1 enzyme. The linearised plasmid DNA was purified, using column kit, and used as templates for transcription of DIG-riboprobe. The reaction conditions were as follows:

- 500 ng-1 µg of linearised plasmid DNA
- 2 µl 10X DTT (Promega)
- 4 µl 5X transcription buffer (Promega)
- 1 µl T3 RNA polymerase (Promega)
- 1 µl DIG nucleotide mix (Roche)
- 1 µl RNAsin
- Water up to 20 µl

The reaction was incubated at 37°C for 2 hours. It was stopped by adding 2 µl of 0.5M EDTA followed by 20 µl DEPC (Diethylpyrocarbonate)-treated water. The RNA probes were precipitated with 10 µl Lithium Chloride (LiCl; 4M) and 250 µl absolute Ethanol for 2 hours at -80°C. The probes were centrifuged at 4°C at maximum speed for 30 minutes. The supernatant was removed and the pellet was washed by vortexing with 70% Ethanol. The sample was centrifuged again and the alcohol was removed and the pellet was left to air dry for 30 minutes. The pellet was resuspended with 50 µl of DEPC-treated water. The probe was denatured for 3minute at 95°C then immediately cooled on ice for 5 minutes. The RNA probe was checked by running 1 µl in parallel with the template on an agarose gel. The probes were diluted in 1:100 in hybridisation buffer. The probes were stored at

either -20°C for short-mid-term storage or -80°C for long-term storage. The following recipe was used to make the hybridisation buffer which was stored at -20°C:

- 50% deionized formamide
- 5% SSC (Sodium Saline Citrate buffer)
- 2% Boehringer Blocking powder
- 0.2% Triton X100
- 50 µg/ml heparin
- 50 µg/ml yeast t-RNA
- 5 mM EDTA

#### **2.4.2 Tissue preparation for *in situ* hybridisation**

E3-5 whole embryos or dissected heads of embryos at E7 and E10 were rinsed in DEPC-treated PBS then fixed in 4% Paraformaldehyde (PFA) in PBS for 2 hours at room temperature or overnight at 4°C. The ears of E5, E7 and E10 chick embryos were then dissected, removing the cartilaginous capsule that surrounds the membranous part of the inner ear. For stages E3-5, a small hole was made, using a syringe needle, at the base of the endolymphatic duct to prevent trapping of solutions. The ear tissue samples were rinsed twice with PBS+0.1% Tween 20 (PBTw) and dehydrated with a dilution series of Methanol (MeOH) (25%, 50% and 75% MeOH with PBTw). The embryos were incubated, with agitation, for 5 minutes in each solution, then finally incubated in 100% MeOH twice for five minutes. Dehydrated samples were stored at -20°C until further use.

#### **2.4.3 *In Situ* Hybridisation on whole-mounts**

Non-radioactive *in situ* hybridisation (ISH) on whole-mount embryos was performed as described in (Ariza-McNaughton and Krumlauf, 2002) with minor modifications. Ear tissue samples were rehydrated using the same MeOH series, but in the reverse order finally ending with two rinses in PBTw. The tissues were treated with proteinase K (10 µg/ml in PBTw) for 5-20 minutes at room temperature, depending on the developmental stage. Following two rinses with PBTw, the tissues were fixed with 4% PFA for 30 minutes at room temperature. The tissues were first incubated in hybridisation buffer for 2 hours at 62°C. The buffer was then replaced with pre-warmed hybridisation buffer containing the RNA probe (200ng-1µg/ml). The tissues were incubated with the probe at 62°C overnight. Two rinses were performed at 62°C with pre-warmed 2X SSC+0.1% Triton-X100 followed by two rinses with 0.2X SSC+0.1% Triton-X100, each for 30 minutes with occasional gentle inversion. The tissues were rinsed twice with

1X KTBT (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM KCl, 0.1% Triton-X100) for 5 minutes at room temperature with agitation then incubated in blocking solution of 10% sheep serum in KTBT for 2 hours at room temperature. The embryos were incubated overnight at 4°C with anti-DIG antibody coupled to alkaline phosphatase (Roche, UK) diluted 1:2000 in the sheep serum/KTBT blocking solution. Extensive rinses were then performed at room temperature in KTBT (3 x 1hour). The tissues were rinsed twice in Alkaline phosphate (AP) buffer (100 mM Tris-HCl pH 9.5, 50 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.1% Tween) at room temperature. The colour reaction was carried out in the dark with AP buffer containing 4.5 µl/ml of NBT (nitroblue tetrazolium, 75mg/ml in 70% dimethylformamide) and 3.5µl/ml of BCIP (5-bromo-4chloro-3-indolyl-phosphate, 50 mg/ml in 100% dimethylformamide). The reaction was regularly monitored and stopped by rinsing twice with 1X KTBT followed by fixation with 4% PFA to stabilize the staining. Samples were stored at 4°C with 4% PFA.

## **2.5 Immunohistochemistry**

Embryos aged E2-E3 or dissected inner ear tissue was fixed in 4% PFA in PBS for 30 minutes to 2 hours at room temperature or at 4°C overnight, depending on the developmental stage.

Following two rinses in PBS, the samples were incubated with a blocking solution (PBS containing 10% goat serum and 0.1% Triton-X100) for 2 hours at room temperature. The blocking solution was removed and samples were incubated with primary antibody diluted with the blocking solution at 4°C overnight. Following 3 x 20 minutes rinses in PBS with 0.3% Triton (PBT), the samples were incubated with secondary antibody diluted in blocking solution (1% goat serum in 0.3% PBT) for 2 hours at room temperature. Following rinses with PBT, some samples were treated with 4',6-diamidino-2-phenylindole (DAPI) (1:500 in PBT) for 20 minutes at room temperature. The tissues were mounted using Vectashield (Vector Laboratories, UK) onto multi-well slides and imaged using an inverted confocal microscope (LSM510; Zeiss) or an Axioplan upright microscope.

The following primary antibodies were used: rabbit anti-Serrate1 (1:100; see Adam et al., 1998) and rabbit anti-Delta1 (Henrique et al., 1997), monoclonal mouse IgG1 anti-hair cell antigen (HCA; a kind gift from Guy Richardson, see (Richardson et al., 1990);(Bartolami et al., 1991)) (supernatant used at 1:1000) and monoclonal mouse IgG2b anti-otoferlin (HCS1; a kind gift of Jeff Corwin, see (Gale et al., 2000) (used at 1:250).

Secondary antibodies included species-specific goat anti-Ig coupled to AlexaFluor 405, 488, 546 or 633, depending on the desired wavelength (1:500, Molecular Probes, UK).

### **2.5.1 Tissue Preparation for cryosections**

Whole embryos were fixed in 4% PFA solution for 2 hours at room temperature or overnight at 4°C with rotation. The PFA solution was removed and the embryos were washed twice in PBS. For cryoprotection, the embryos were incubated in a 15% sucrose solution (with PBS) at room temperature for 2 hours with rotation then in a 30% sucrose solution overnight at 4°C. The embryos were dissected below the ear for embedding. The dissected heads were embedded in a 1.4% Ultra Pure™ low melting point agarose (Invitrogen) gel. The agarose was made up in 18% sucrose-PBS and was heated to 140°C to dissolve, then allowed to cool to 37°C. The agarose solution was poured into moulds and the embryonic heads were immersed completely into the agarose. The heads were orientated such that the anterior was facing down. The samples were then incubated overnight at 4°C for the agarose to set. The sample blocks were rapidly frozen by immersion in isopentane that had been cooled to -50°C, using liquid nitrogen. The moulds were removed and the frozen blocks were attached to cryostat chucks using OCT embedding matrix (Solmedia). The attached blocks were incubated for 15 minutes at -25°C. Transverse sections (20 µm thickness) were cut and collected on Superfrost slides.

## **2.6 Live cell imaging of inner ear culture and DAPT treatment**

Embryos were electroporated with the RCAN-Hes5-d2EGFP or RCAS(B)-EGFP construct at E2-E2.5, and returned to the incubator for 6 days. The transfected inner ear were dissected out at room temperature and maintained in a 30mm Petri dish with DMEM/F-12 (without phenol red; Invitrogen) at 37°C, 5% CO<sub>2</sub>. To block Notch signalling, I replaced the control medium with DMEM/F-12 containing 20µM of DAPT (N-[N-(3, 5-difluorophenacetyl)-1-ala-nyl]-S-phenylglycine t-butyl ester; Calbiochem, UK), an inhibitor of gamma-secretase activity (Dovey et al., 2001; Geling et al., 2002). In one experiment, I used an MZ16F Leica stereomicroscope connected to a CCD camera (Orca) to capture fluorescent images of free-floating cultures transfected with either RCAN-Hes5-d2EGFP or RCAS(B)-EGFP. The cultures were treated with DAPT at time 0 and pictures were taken at 3, 6 and 9 hours.



Time-lapse confocal imaging of inner ear samples transfected with RCAN-Hes5-d2EGFP was performed using an inverted Nipkow spinning disc confocal microscope (Ultraview ERS, Perkin Elmer, UK) connected to an Orca ER CCD camera. The cultures were placed in a 35mm glass bottom dish (Mattek Corporation, USA), immobilised with a platinum “harp” and maintained at 37°C and 5% CO<sub>2</sub> with an incubation chamber. Series of 14 to 16 z-optical sections were collected every 20 minutes using a 20X Neofluar or a 40X Neofluar oil-immersion lenses. Fluorescence levels were then analysed using Lucida software (Andor). First an average projection of the z-stack at each time point was made. Then two regions of interest (ROIs) were selected: One ROI encompassing the majority of the labelled region and a second smaller region within the labelled region, chosen at random. The background levels, in untransfected regions of the tissue, were subtracted from each ROI. The fluorescence levels at each time point were then normalised by dividing with the maximum intensity value of this ROI over the entire time-course.

Time-lapse confocal imaging of inner ear samples transfected with pT2K-Hes5-d2EGFP and pT2K-TomatoNLS was performed using an inverted confocal microscope (LSM510; Zeiss). Cell-tak™ (BD Biosciences) was used to stick the tissue to the glass bottom dish (instead of a platinum harp) to avoid movement during imaging. The glass was coated with a solution of 2 µl of cell-tak diluted in 20 µl 0.1M NaHCO<sub>3</sub>. This was incubated at room temperature for 30 minutes. Excess solution was removed and washed twice with culturing medium. The inner ear tissue was placed in the centre of the glass dish. The solution was removed and the tissue was allowed to stick to the cell-tak for a few seconds. The culture medium was replaced slowly. The culture was incubated at 37°C, 5% CO<sub>2</sub> overnight before imaging.

# 3

## CHARACTERISATION OF A FLUORESCENT REPORTER OF NOTCH ACTIVITY

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### **3.1 A comparative study of two reporters of Notch activity: Hes5-d2EGFP and CBF-Venus d2YFP**

#### **3.1.1 *Reporters of Notch activity***

During inner ear development, Notch signalling plays different roles, such as regulation of otic specification and neurogenesis, early formation of the sensory patches and hair cell differentiation within these patches. In order to investigate the spatio-temporal patterns of Notch activity during inner ear development, in living tissue, genetically encoded fluorescent reporters were used in this study. Such reporters have been extensively used in cell lines and various tissues to monitor Notch activity, but not in the inner ear.

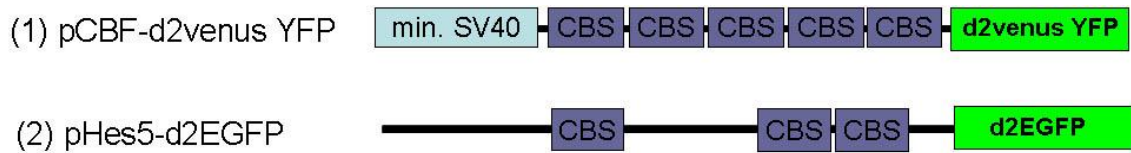
The two main types of reporters of Notch activity are composed of either multiple repeats of CSL binding sites, or endogenous *cis*-regulatory elements of Notch target genes (such as members of the Hes family) which drive the expression of a reporter gene (Basak and Taylor, 2007; Kohyama et al., 2005; Ong et al., 2006). In this study, I initially tested two reporters representative of each category: pCBF-d2VenusYFP and pHes5-d2EGFP. The pCBF-d2VenusYFP reporter contains a minimal SV40 promoter upstream of five consecutive repeats of the CBF binding sites (CBS) found in the promoter region of Notch target genes (Ong et al., 2006). These regulatory elements drive expression of a destabilised form of Venus Yellow Fluorescent Protein (d2Venus YFP). The pHes5-d2EGFP reporter consists of

a 0.76 kb *Hes5* cis-regulatory element, from the mouse *Hes5* promoter, originally characterised and described by Takebayashi et al. (1995), that drives the expression of a destabilised variant of an enhanced green fluorescent protein (d2EGFP) which has been optimised for brighter fluorescence (Excitation maximum=488nm; emission maximum=507nm) (see figure 3.1).

For investigating temporal variations in levels of Notch activity, it is important that the reporter gene in these two constructs is relatively short-lived. Hence the destabilised d2Venus YFP and d2EGFP were constructed by adding residues 422-461 of mouse ornithine decarboxylase (MODC) to the C-terminus of YFP/EGFP. This region contains a PEST (proline (P), glutamic acid (E), serine (S) and threonine (T)) amino acid sequence that targets the proteins for degradation via the ubiquitin/proteasome system and results in rapid protein turnover. The d2EGFP has a half-life of approximately 2 hours. This was measured by fluorescence intensity of cells treated with cycloheximide (protein synthesis inhibitor) and Western blot analysis (Li et al., 1998). This allows for greater temporal resolution compared to the stable wild-type GFP, which has a half-life of approximately 26 hours (Corish and Tyler-Smith, 1999).

Reporters of Notch activity have previously been used in different animal models and tissues such as the mouse CNS and kidney (Ohtsuka et al., 2006; Ong et al., 2006) and chick retina (Nelson et al., 2006). A reporter plasmid containing the luciferase gene under the control of the 5'-region of the *Hes5* gene was able to drive expression in undifferentiated neural cells prepared from E10.5 mouse (Takebayashi et al., 1995). Studies using a *Hes1* reporter, in which ubiquitinated luciferase was expressed under the control of the *Hes1* promoter, showed that *Hes1* is expressed in an oscillatory manner during somitogenesis (Masamizu et al., 2006) and with neural progenitors (Shimojo et al., 2008). Therefore reporters based on the Notch target genes, *Hes1* and *Hes5*, enable the visualisation in living tissue of the spatio-temporal pattern of Notch signalling.

The pHes5-d2EGFP reporter has been previously used in the chick retina (Nelson et al., 2006). The results from this study showed that this reporter can be used to reveal endogenous spatial patterns of active Notch signalling during retinal ganglion cell differentiation (Nelson et al., 2006). However neither the pCBF-d2VenusYFP nor the pHes5-d2EGFP reporters have ever been tested within the chick inner ear.



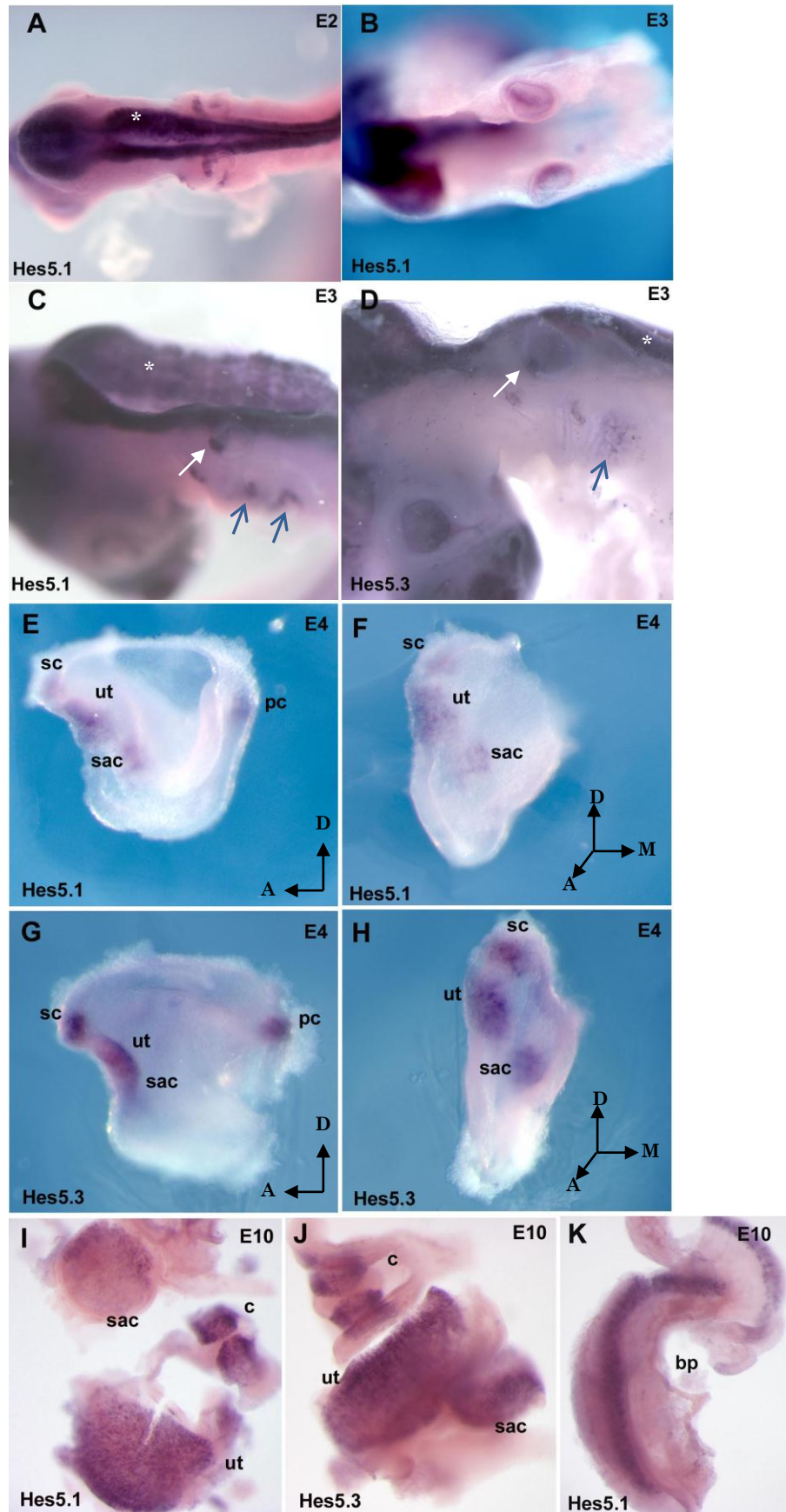
**Figure 3.1 Reporters of Notch activity** Schematic maps (not to scale) of (1) pCBF-d2venus YFP reporter which contains a minimal SV40 promoter upstream of five consecutive repeats of the CBF binding sites (CBS) that drive the expression of a destabilised venus YFP and (2) pHes5-d2EGFP reporter which contains a 0.76 kb Hes5 *cis*-regulatory element, from the mouse Hes5 promoter, that drives the expression of a destabilised EGFP.

### 3.1.2 Expression pattern of *Hes5.1* and *Hes5.3* in the chick inner ear

It has been shown that *Hes5* genes are direct effectors of Notch activity during neurogenesis (de la Pompa et al., 1997; Fior and Henrique, 2005; Hatakeyama et al., 2004; Kageyama and Ohtsuka, 1999). These genes encode basic helix-loop-helix proteins of the Orange subtype (bHLH-O) that repress the expression of other bHLH proneural proteins (reviewed by Bertrand et al. (2002).

There are at least 6 members of the *Hes* (Hairy and Enhancer of Split) gene family present in the chicken genome. Three of these genes encode proteins with strong homology to the mammalian Hes5: *Hes5.1*, *Hes5.2*, and *Hes5.3*. The other two genes encode proteins related to the mammalian Hes6 protein: *Hes6.1* and *Hes6.2* (Fior and Henrique, 2005). Another member of the Hes family of proteins, *Hairy1*, is thought to be the homologue of the mammalian *Hes1* gene.

In the chicken otic placode, *Hairy1* is expressed in the non-neural domain and in a pattern that does not overlap with that of any Notch ligands. In contrast, all *Hes5* genes are present in the anterior neurogenic patch, where neuroblasts are selected by lateral inhibition and the Notch ligands Delta1 and Serrate1 are expressed (Abello et al., 2007; Daudet et al., 2007). The neurogenic domain is also thought to give rise to the majority of the prosensory domains that will eventually form sensory epithelia. This suggests that in the chicken inner ear at least, the primary effectors of Notch activity are the *Hes5* genes. To verify this, I characterised the expression pattern of *Hes5.1* and *Hes5.3* during chick inner ear development, using *in situ* hybridisation to detect their mRNA. Daudet et al. (2007) found that *Hes5.2* expression was relatively weak as compared to that of the other *Hes5* genes in the chick inner ear, so it was omitted from this study.



**Figure 3.2 Whole mount *in situ* hybridisation analysis of chicken Hes5.1 and Hes5.3 in the developing chick inner ear.** (A-D) At E2-3 both Hes5.1 and Hes5.3 are expressed in the anterior region of the otic cup (white arrows), the neural tube (white asterisk) and the brachial arches (blue arrow). (A) E2 embryo Hes5.1 expression; dorsal view with anterior on the left. (B) E3 embryo Hes5.1 expression; dorsal view with anterior on the left; neural tube removed. (C) E3 embryo Hes5.1 expression; lateral view. (D) E3 embryo Hes5.3 expression; lateral view. At E4, Hes5.1 and Hes5.3 is expressed in the sc, ut, sac and pc (E and G; medial view, F and H; side view). (I,J,K) E10 dissected utricle, saccule and crista and basilar papilla. At E10 Hes5.1 and Hes5.3 is expressed in all 6 of the sensory patches (NB: 2 cristae not shown). sc; superior crista, pc; posterior crista, lc; lateral crista, ut; utricle, sac; saccule, bp; basilar papilla.

The results showed that at stage E2-E3 (HH 12), there is only one patch of *Hes5* expression in the otic cup, in the anterior/neurogenic domain (Figure 3.2 A-D). At E4, the otic cup has transformed into an otocyst in which four distinct regions of *Hes5* expression are present. Their size and position suggest that these correspond to the superior crista (sc), the saccule (sac), the utricle (ut) and the posterior crista (pc). When looking at the otocyst from the medial view (E and G), only 2-3 patches of *Hes5* expression can be identified, but by turning the otocyst on its side (F and H), it becomes clear that there are in fact four distinct patches at this stage of chick inner ear development. At E5, five distinct patches of *Hes5* expression: the superior crista (sc), the saccule (sac), the utricle (ut), the posterior crista (pc) and the lateral crista (lc) (not shown here, see Chapter 4, Figure 4.9) can be identified. In the basilar papilla (bp), *Hes5* genes are either not present or expressed at very low levels at this stage. At E10 (Figure 3.2 I-K), *Hes5* is expressed in all of the sensory patches. In the panels I and J, only one crista is shown in each case which is representative of all three. There is also *Hes5* expression in the basilar papilla (K). At least 3 embryos were processed for each condition and each showed the same expression pattern.

These results indicated that there is no apparent difference between *Hes5.1* and *Hes5.3* expression patterns, suggesting that both genes are downstream effectors of Notch signalling during chick inner ear development. The results also showed that the spatio-temporal pattern of both *Hes5.1* and *Hes5.3* expression maps to prosensory domains and mature sensory epithelia where the Notch ligands *Serrate1* and *Delta1* are expressed (Adam et al., 1998; Cole et al., 2000; Morrison et al., 1999). Therefore *Hes5* genes are likely direct targets and effectors of ligand-dependent Notch signalling throughout inner ear development. These results provided a useful reference to determine which of the Notch reporters would best reflect the endogenous pattern of Notch signalling during inner ear development.

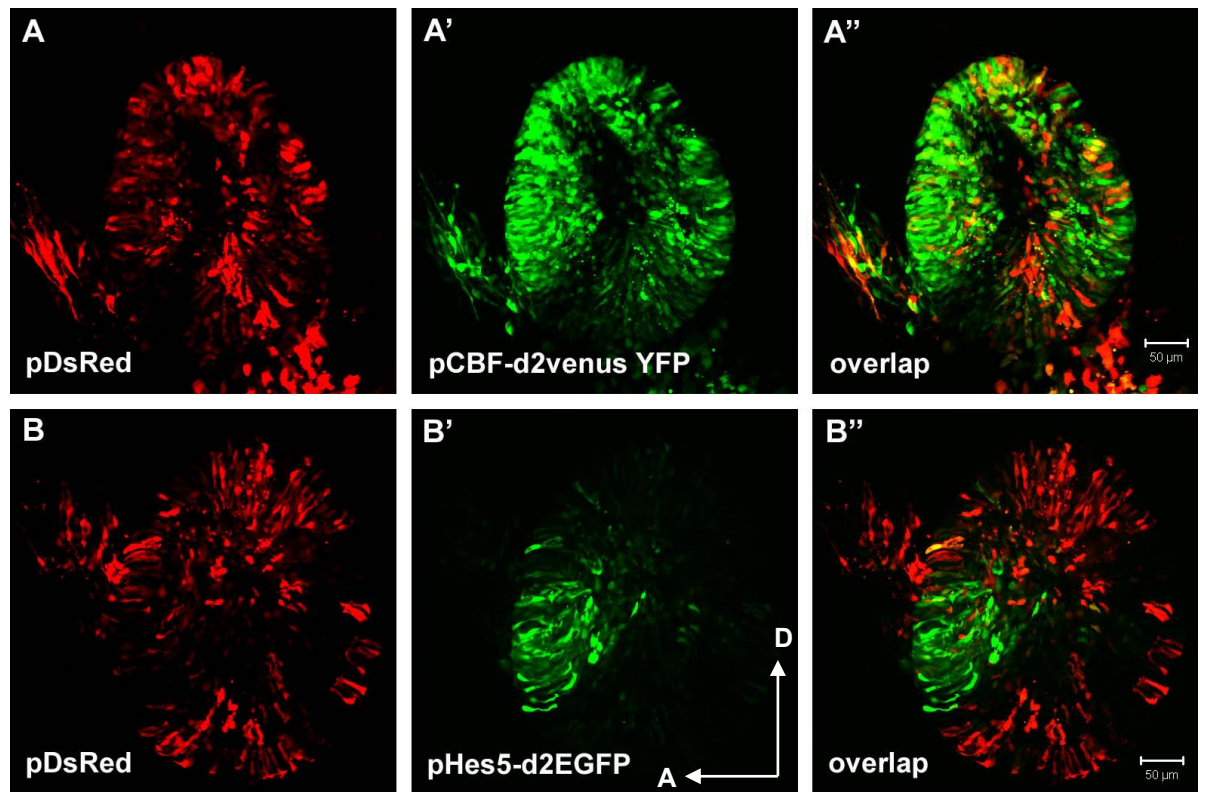
### **3.1.3 The *pHes5-d2EGFP* is more specifically active in the anterior region of the otic cup than the *pCBF-venusd2YFP* reporter**

To test the reporters, *in ovo* electroporation was used to transfect cells of the embryonic chick inner ear at 2 days of incubation (HH13-17), when the otic placode has invaginated to form the otic cup. At this stage, the otic cup can be easily filled with the DNA solution and electroporated efficiently without compromising embryo survival.

Following electroporation, the embryos were incubated for a further 20 hours then fixed and analysed at E3 by confocal microscopy. At this stage, Notch-mediated lateral inhibition regulates the production of neuroblasts (the precursors for auditory and vestibular neurons) that delaminate from the anterior/neurogenic region of the otic cup (Henrique et al., 1995). This is reflected by a strong and restricted expression of the Notch ligand *Delta1* and the Notch effectors of the *Hes5* family in this domain (Daudet et al., 2007).

The pCBF-d2VenusYFP reporter was tested first. To identify all transfected cells, a control plasmid containing a red fluorescent protein (RFP) under a constitutive CMV promoter, pDsRed, was co-transfected. In the electroporated otic cup, transfected cells were clearly recognizable by their red fluorescence. However their distribution was mosaic and the levels of red fluorescence varied greatly from one cell to another. These variations were probably a consequence of variations in the number of plasmid copies transfected per cell. Unexpectedly, the pattern of activation of the pCBF-d2VenusYFP reporter was very similar to that of the pDsRed plasmid. The intensity of YFP fluorescence was not specifically increased in the anterior region of the otocyst in any of the 11 successfully transfected specimens that were analysed (see figure 3.3 A-A”).

The pHes5-d2GFP reporter was then tested under the same conditions. In contrast to the activation pCBF-d2VenusYFP reporter, the levels of activation of the pHes5-d2GFP reporter were clearly greater in the anterior region of the otic cup, where Notch signalling and *Hes5* gene expression normally occurs (n = 20 out of 22 embryos) (See figure 3.3 B-B”). Activation of the reporter was not totally restricted to the anterior patch, but only a few cells outside of this region showed high levels of green fluorescence. In the neurogenic region, cells displaying very low levels of red fluorescent proteins could show high levels of green fluorescence; this suggests that activation of the reporter was strong even in cases where the efficiency of transfection may have been low. In pCBF-d2VenusYFP transfected otocysts, green fluorescence was observed in the neuroblasts that had delaminated from the otocyst (See figure 3.3 A’). In contrast, pHes5-d2EGFP transfected neuroblasts were devoid of green fluorescence. Since neuroblasts derive from the anterior region of the otic cup and escape Notch activation, this observation suggest that activity of the pHes5-d2EGFP reporter is rapidly switched off when cells are no longer experiencing Notch activation.



**Figure 3.3 CBF-d2venusYFP and Hes5-d2EGFP reporter activation.** Inner ears were co-electroporated with pCMV-DsRed (**A**, **B**) and either reporter pCBF-d2venus YFP (**A'**) or pHes5-d2EGFP (**B'**) at E2-E2.5 and harvested 20 h later. The Hes5-d2EGFP reporter is activated in the anterior region (**B'**) ( $n = 20$ ), where there is Notch activity at this stage of chick inner ear development whereas the CBF-d2venus YFP reporter has a more widespread activation pattern (**A'**) ( $n = 11$ ). Scale bar = 50 $\mu$ m (applies to all panels).



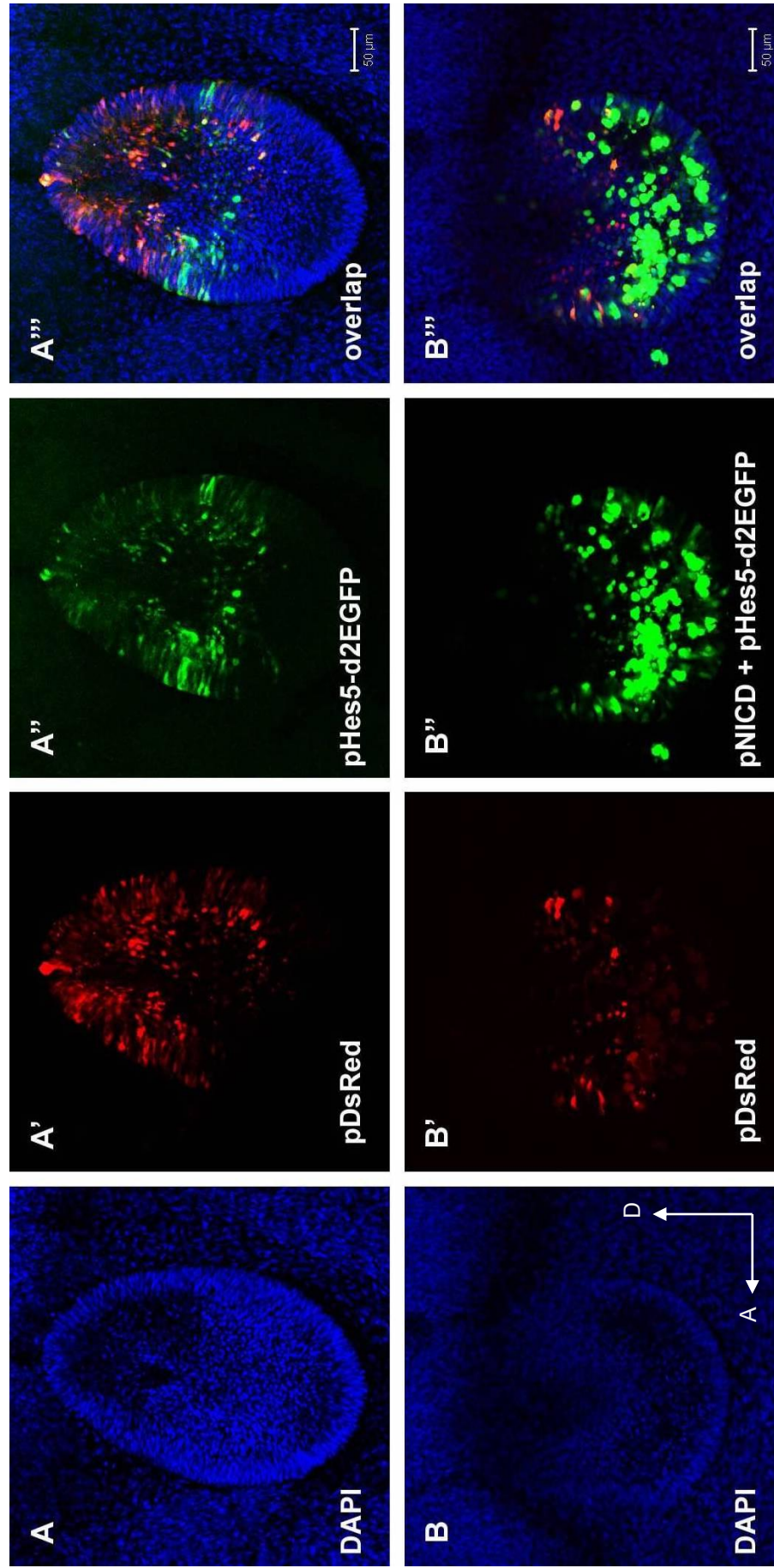
In conclusion, the pHes5-d2EGFP reporter containing cis-regulatory elements of the mouse Hes5 promoter gave a more accurate read-out of endogenous *Hes5* expression in the otocyst than the pCBF-d2Venus YFP reporter, containing multiple repeats of CSL binding sites. This confirms the importance of the promoter context and organisation of CSL binding sites in the regulation of the expression of Notch target genes (Cave et al., 2005; Ong et al., 2006). Therefore, the pHes5-d2EGFP reporter was used for the rest of this study.

## **3.2 The pHes5-d2EGFP reporter is sensitive to variations of Notch activity**

### ***3.2.1 Overexpression of chick Notch1 intracellular domain leads to a strong increase in reporter activity***

To test whether the pHes5-d2EGFP reporter would respond to an artificial increase in Notch activity, I used the pNICD construct that drives constitutive expression of the chicken Notch1 Intra-Cellular Domain (NICD). When Notch receptors are activated by DSL ligands, the NICD is cleaved by the  $\gamma$ -secretase complex and translocates to the nucleus where it acts as a transcription factor to activate the expression of Notch target genes, such as *Hes5*. Therefore, a common way to mimic an activation of the Notch pathway is to overexpress the NICD (Daudet and Lewis, 2005; Ong et al., 2006).

The otic cup was co-transfected with the pHes5-d2EGFP reporter and pNICD, along with pDsRed as a marker for transfection. The activation pattern of the reporter was compared to the control condition, in which the pNICD construct was omitted. In the control embryos, reporter activation was restricted to the anterior region of the otic cup, as seen in the initial experiments in section 3.1. Overexpression of NICD led to a strong and widespread activation of the reporter in 10 out of 17 embryos. Strong induction of fluorescence was also observed in the most dorsal and posterior regions of the otic cup, which are normally devoid of Notch activity (See figure 3.4). These results confirmed that the pHes5-d2EGFP reporter is sensitive to an artificial overexpression of Notch activity and thus could be used to monitor endogenous Notch activity.



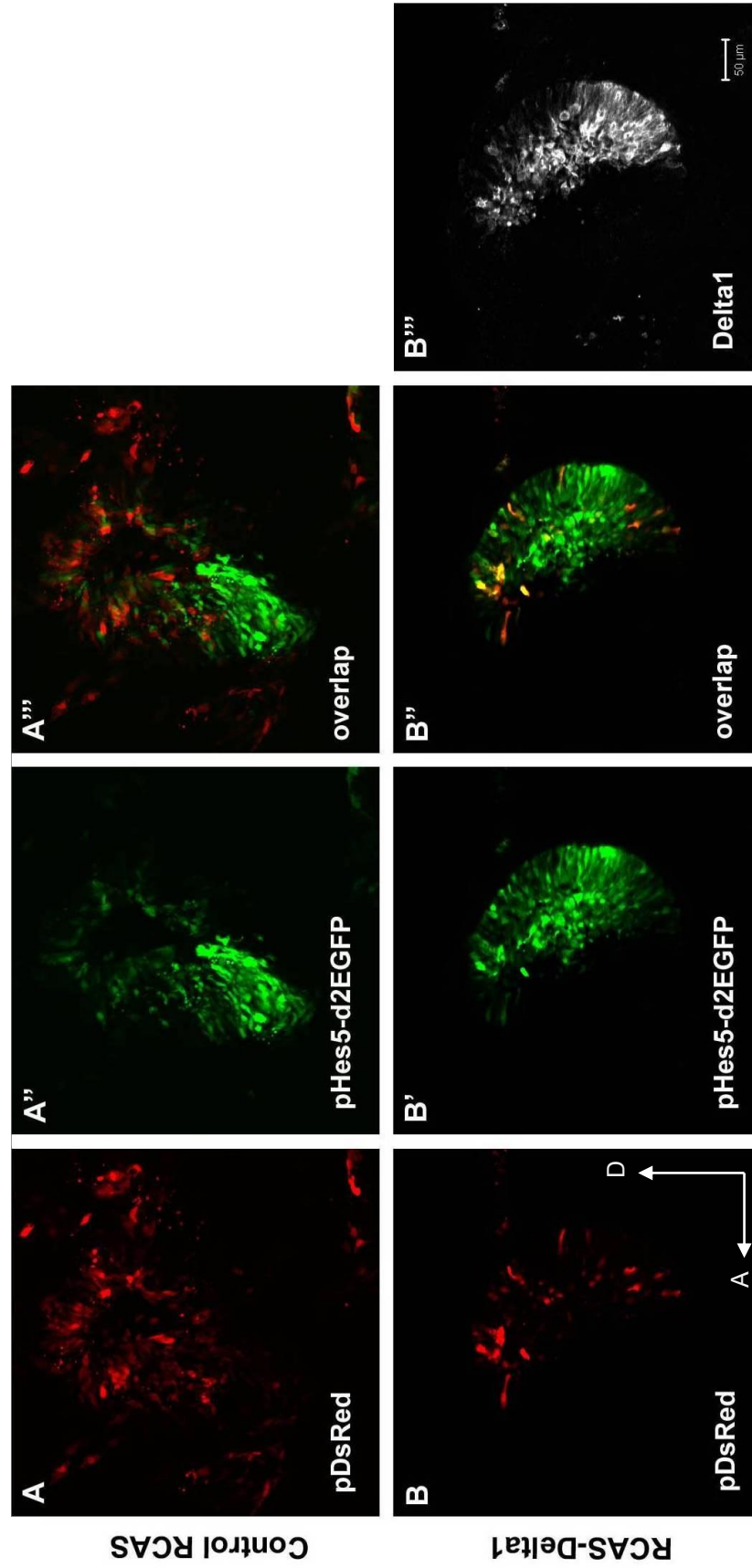
**Figure 3.4 Overexpression of chicken NICD (pNICD) strongly activates the pHes5-d2EGFP reporter.** Inner ears were co-electroporated with either pDsRed (A') and pHes5-d2EGFP (A'') or pDsRed (B'), pHes5-d2EGFP (B'') and pNICD at E2. (A'') shows the same pattern of activation as seen previously. (B'') shows strong ectopic activation of the reporter (n = 10 in 3 experiments). A and B show DAPI staining. Scale bar = 50μm (applies to all panels)

### **3.2.2 Overexpression of the Notch ligand, Delta1, leads to a strong increase in reporter activity**

To further confirm the reporter's sensitivity to Notch signalling, another method was used to artificially induce Notch activity in the otic cup. The Notch ligand Delta1 was overexpressed using a retroviral, RCAS (Replication Competent ASLV long terminal repeat with a Splice acceptor site) vector, which was expected to activate Notch signalling in transfected regions. At otic placode and cup stages, Delta1 expression is normally restricted to the anterior neurogenic patch. It is expressed in a "salt-and-pepper" pattern by neuroblasts that delaminate from the otic epithelium (Adam et al., 1998).

The otic cup was co-transfected with an RCAS-Delta1 construct with the pHes5-d2EGFP reporter and pDsRed. Similarly to the overexpression of NICD, the reporter was strongly activated in regions where Delta1 is overexpressed and where Notch is not normally active. In figure 3.5, there is no reporter activity in the anterior domain because the otic cup was transfected in the posterior region, where Delta1 is normally absent. Ectopic Delta1 expression in this region resulted in a strong increase in fluorescence of the reporter. As confirmed by immunocytochemistry, strong expression of the Delta1 protein was induced in transfected regions (n = 10 from 3 separate experiments).

Altogether, these results showed that the pHes5-d2EGFP reporter is active in the otic cup domain where ligand-dependent Notch signalling occurs, and is sensitive to experimentally induced increases in the levels of Delta1 or Notch1 activity. The results also suggested that the reporter could be responsive to endogenous variations of Notch activity resulting from Delta1/Notch1 signalling in the inner ear at later stages of development.



**Figure 3.5 Overexpression of the active form of the chicken Delta1 (RCAS-Delta1) strongly activates the pHes5-d2EGFP reporter.** Inner ears were co-electroporated at E2 with either pDsRed (A), pHes5-d2EGFP (A') and an empty RCAS construct as a control (A-A'') or pDsRed (B), pHes5-d2EGFP (B') and RCAS-Delta1 (B-B''). (B''') RCAS-Delta1 electroporated embryos were stained with anti-Delta1 (white) (n = 2). Scale bar = 50μm (applies to all panels).

# 4

## RCAN AND TOL2 TRANSPOSON ENABLE STABLE INTEGRATION OF THE HES5-D2EGFP REPORTER IN THE DEVELOPING INNER EAR

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The previous results indicated that the pHes5-d2EGFP reporter could be used to monitor Notch activity in living cells at early stages of development of the chicken inner ear. However, it would not have been possible to use this reporter plasmid to analyse the pattern of Notch activity at later stages of development. In fact, as transiently transfected cells divide and development proceeds, plasmid DNA is gradually degraded. At the stage of hair cell production, starting at about E5 in vestibular sensory patches, a very low amount of reporter plasmid would be left in the progeny of transfected cells.

To circumvent this problem, I decided to clone the Hes5-d2EGFP reporter elements into new vectors that could lead to stable integration of the reporter into the genome of transfected cells: the RCAN retrovirus and the Tol2 transposon.

## **4.1 RCAN, a replication competent avian retrovirus, enables stable integration of the Hes5 reporter in the developing chick inner ear**

### **4.1.1 Introduction to the RCAS system**

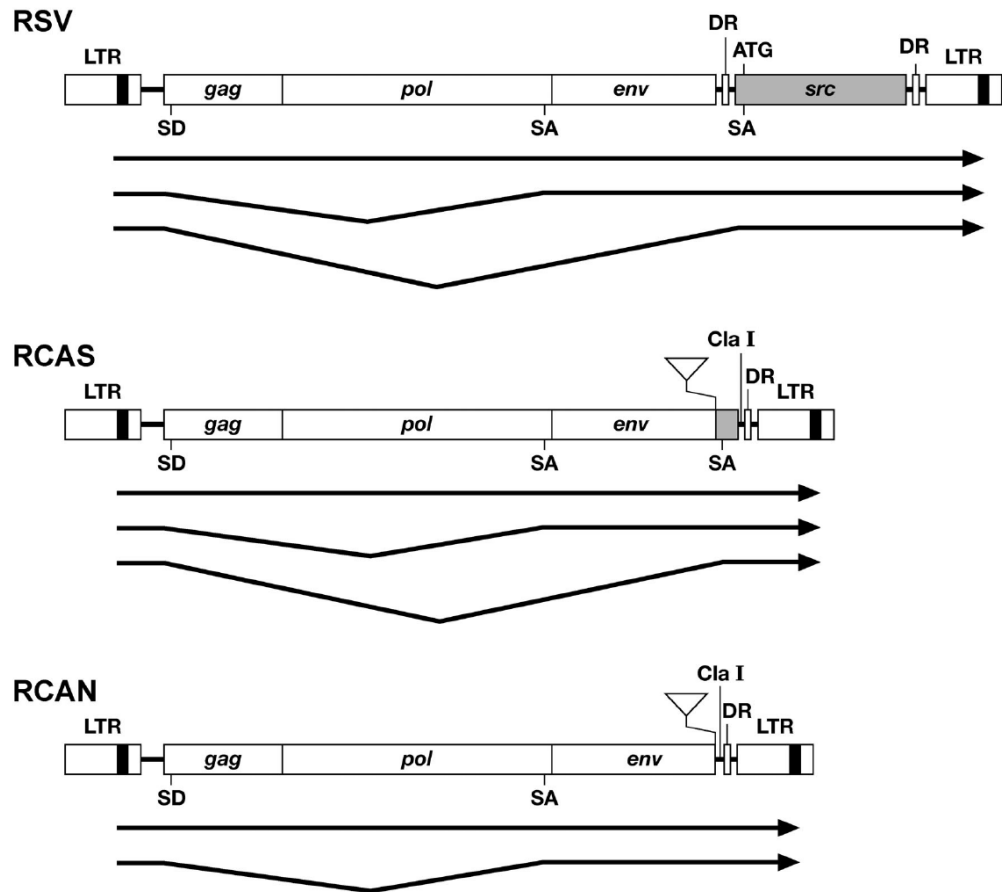
The RCAS retroviral vectors are derived from the SR-A strain of Rous sarcoma virus (RSV), a member of the avian sarcoma-leukosis virus (ASLV) family. The RSV has maintained its viral genes (*gag/pol* and *env*) whilst acquiring an oncogene, *src*, thus remaining replication-competent retroviral vectors. To create the RCAS vector, the *src* oncogene was deleted and a unique restriction site (ClaI) was inserted in its place (Hughes et al., 1987). Other exogenous genes/sequences (up to 2.5kb long) can therefore be substituted for *src* in the ClaI site adjacent to the splice acceptor site of RCAS. These vectors will replicate in chicken cells and in some cases, if appropriately chosen, can infect, but not replicate in some mammalian cells (the amphotrophic versions of the RCAS vectors - for more info see <http://home.ncifcrf.gov/hivdrp/RCAS/overview.html>). Empty vectors (vectors carrying no exogenous gene) are equivalent to natural viruses such as RAV (Rous-associated virus) and other than virus replication, show no biological effects on the infected cell, thus they can be used for controls.

In order for a retrovirus to infect a cell, there needs to be a specific interaction between the envelope glycoprotein on the surface of the virus and the cognate receptor on the surface of the cell. The ASLV family of viruses has five primary envelope types: A, B, C, D and E which recognise 3 distinct cellular receptors A, C and B/D/E that are located on the surface of the chicken cells. In order for the virus to propagate, the cells must have the appropriate receptor and the chickens used must not already be infected with ASLV. *Env* in subgroup A is most conveniently used as a vector. Cells cannot be infected with a virus belonging to the same subgroup, but can be infected with more than one virus if they belong to a different subgroup. This phenomenon is known as ‘interference’ (reviewed in Hughes, 2004).

The replication/expression level is determined by two components of the vector: the LTR (long terminal repeat) and the *pol* region. Expression of the spliced message for the inserted gene is driven by the viral LTR. The promoter in the ASLV LTR induces high levels of gene expression in avian cells. The choice of *pol* region also affects replication and expression in avian cells, but not in mammalian cells. The widely used, RCASBP (Bryan

Polymerase), is a derivative of RSV produced by substituting the *pol* region from the Bryan high-titer strain of RSV. This virus replicates about ten times better than RCAS in chickens. In addition to the RCAS and RCASBP, there is also RCOS and RCOSBP, RCOS being the least efficient in terms of replication. A parallel family to the RCAS, the RCAN (Replication-Competent, ASLV LTR, No splice acceptor) vector, can be used to express a gene from an appropriate internal promoter that will retain its tissue specificity (Petropoulos et al., 1992). Hence, this is the particular type of vector that was selected for regulated expression of d2EGFP from the Hes5 promoter. Firstly, the Hes5-d2EGFP insert was amplified by PCR from the pHes5-d2EGFP plasmid, using primers containing ClaI sites. This insert was then subcloned into the ClaI site of the RCAN construct (Figure 4.1).





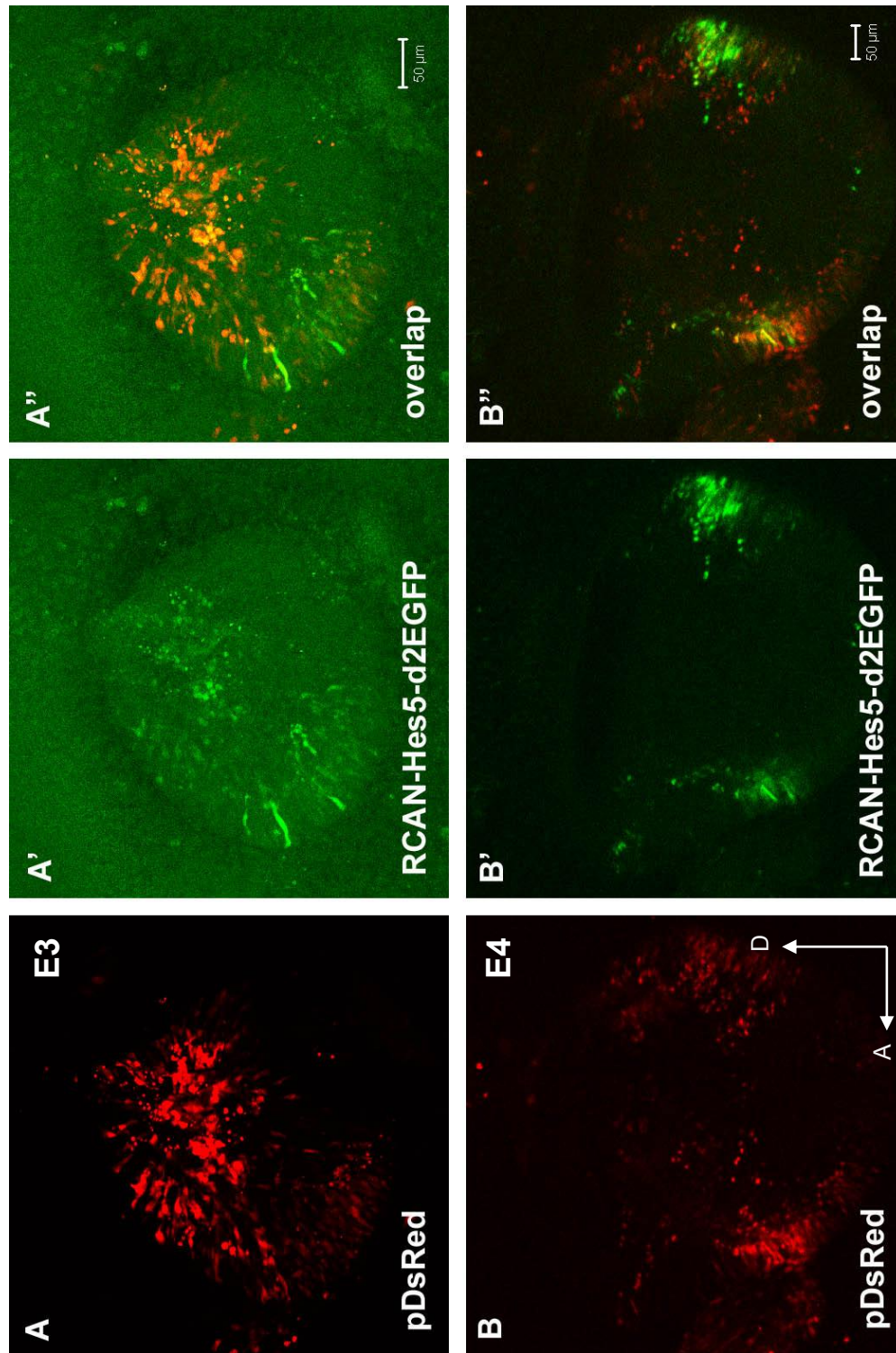
**Figure 4.1 Schematic diagrams of RSV, RCAS and RCAN.** The diagrams show how the viral DNA genomes are organised, the location of the genes (*gag*, *pol*, *env*), the direct repeat (DR), the splice donor (SD) and the splice acceptor (SA) sites. The full-length and the spliced RNAs produced from each viral DNA are indicated below these diagrams. In RCAS the *src* gene has been deleted and replaced by a *Cla*I site, but there is a small segment of the *src* gene remaining which carries the *src* splice acceptor. In RCAN, this segment has been deleted (Not to scale) (adapted from review by Hughes (2004)).

#### **4.1.2 *In ovo* electroporation of RCAN-Hes5-d2EGFP enables stable integration of the Hes5 reporter in the developing chick inner ear**

The RCAS system has been used in previous studies for testing gene function in the embryonic chicken inner ear (Eddison et al., 2000; Morgan and Fekete, 1996; Stevens et al., 2003) and for lineage-tracing analysis with replication-defective retroviruses (Sato and Fekete, 2005). The production of RCAS virus particles is usually achieved through transfection of chicken cell lines with a proviral DNA. The viral particles produced by transfected cells are collected and concentrated by ultracentrifugation, then used for infection *in ovo*. However, it has been shown that *in ovo* electroporation of RCAS proviral DNA can also lead to stable integration of foreign transgenes in the inner ear (Bird et al., 2010). Therefore I reasoned that transfection with RCAN-Hes5-d2EGFP proviral DNA could lead to a stable integration of the reporter into cells of the inner ear. In order to verify this, the otic cup of E2 chick embryos was electroporated with the RCAN-Hes5-d2EGFP plasmid and then allowed to develop until late developmental stages. Parallel experiments were performed with an RCAS plasmid driving constitutive expression of EGFP (RCAS-EGFP) as a control. Twenty-four hours after electroporation, there was very faint activation of the reporter in the anterior region of the otocyst when compared to the levels of fluorescence obtained with electroporation of the pHes5-d2EGFP plasmid (compare panel A' in Figure 4.2 with panel B' in Figure 3.3). At E4, reporter fluorescence was stronger than at E3, and clearly seen in two restricted patches, one anterior and one posterior (see figure 4.2 B'). These regions most likely identify the prosensory domains as defined by the expression of markers such as Sox2, BMP4, and the Notch ligand Serrate1/Jagged1. It also corresponds to the regions in which Hes5 genes are expressed.

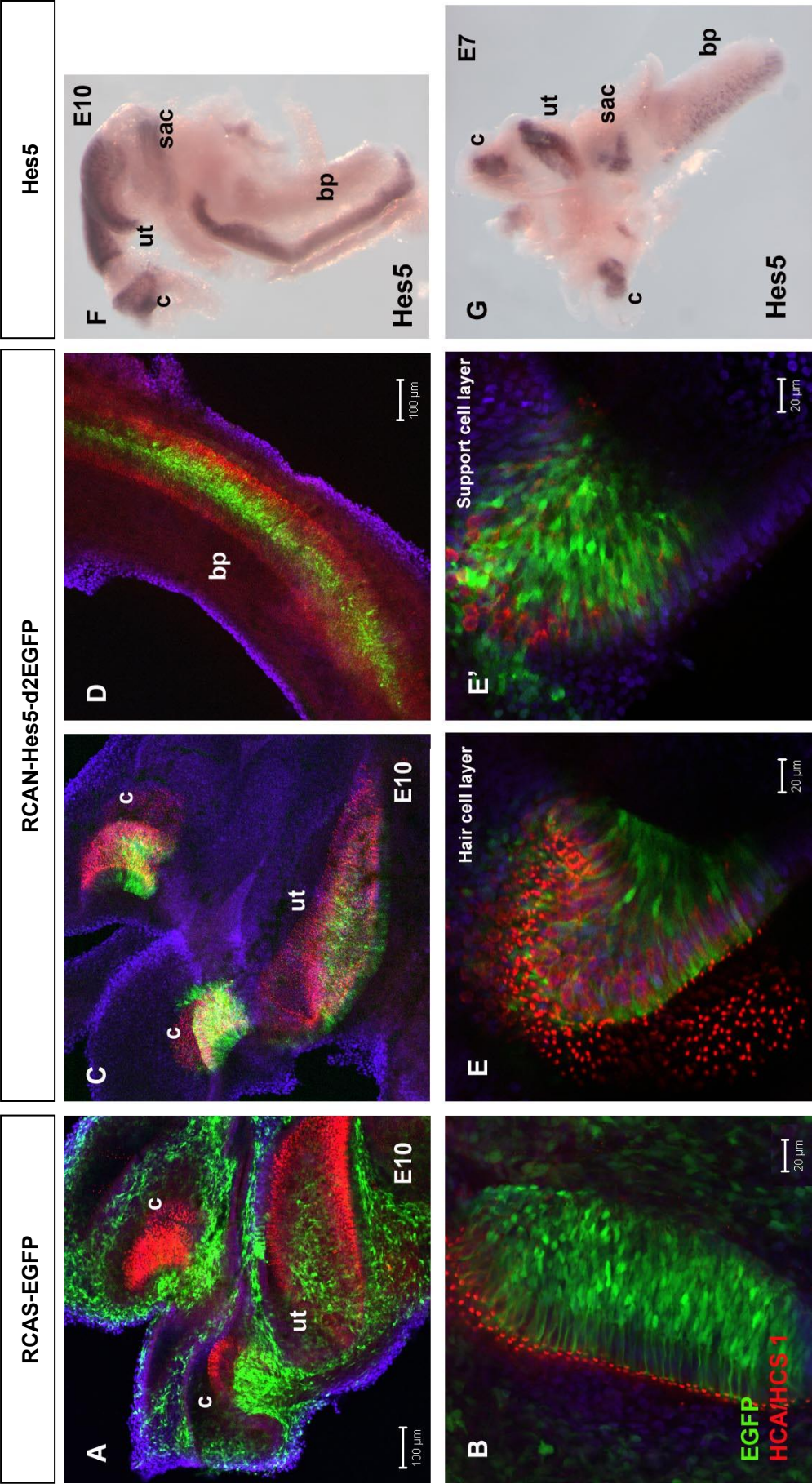
By E10 the sensory patches are well developed and contain a large number of hair cells. The ears transfected with the control RCAS-EGFP construct exhibited EGFP fluorescence throughout the inner ear, in both the sensory and non-sensory regions (see Figure 4.3 A and B). By contrast, the pattern of EGFP fluorescence was confined to the sensory patches in samples electroporated with the RCAN-Hes5-d2EGFP reporter (see Figure 4.3C-E'). The levels of fluorescence were always stronger in the sensory epithelia of the crista than in the utricle, saccule, and basilar papilla. Examination of these samples at high magnification and following immunostaining with hair cell and supporting cell markers showed that EGFP fluorescence was restricted to progenitor and supporting cells occupying the basal layer of the sensory epithelium, and excluded from differentiated hair cells. Again, this

pattern of fluorescence fitted very well with the endogenous pattern of expression of the Hes5.1 and Hes5.3 genes at the same developmental stage (see figure 4.3 E and F). These data demonstrated that electroporation of the RCAN-Hes5-d2EGFP proviral DNA at early stages of otic development could result in stable integration of the Hes5-d2EGFP reporter in the inner ear. Therefore this reporter can be used to monitor spatio-temporal activation patterns of Notch during chick inner ear development.



**Figure 4.2 RCAN-Hes5-d2EGFP reporter activation pattern at E3 and E4.** Inner ears were co-electroporated with pDsRed (**A,B**) and RCAN-Hes5-d2EGFP (**A', B'**) at E2 and harvested 24hrs (**A-A''**) and 48hrs (**B-B''**) later. Activation of this reporter occurs in the anterior region of the otic cup, but is weak (**A'**). When left a further 24hrs two distinct patches in the anterior and posterior regions of the otic vesicle can be seen. Images are representative of at least 3 otocyst at each stage. Scale bar = 50μm (applies to all panels). Note: An increase in the laser power was required in order to detect the weak signal at E3 so hence the background levels appear high.



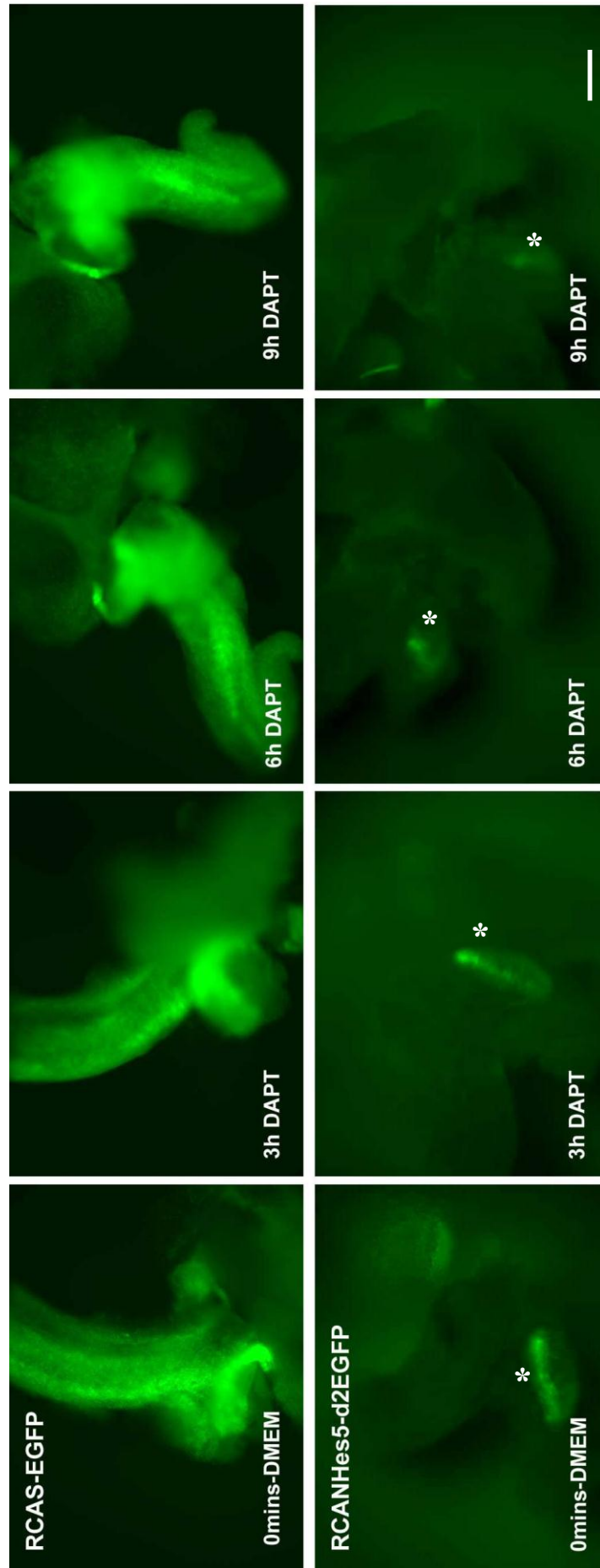


**Figure 4.3 RCAN-Hes5-d2EGFP reporter activation pattern at E10.** Inner ears were electroporated with either RCAS-EGFP (control) or RCAN-Hes5-d2EGFP at E2 and harvested after 8 days, at E10 and were then stained with anti-HCS1 (red), anti-HCA (red), anti-HCS1 (red), anti-HCA (red) and DAPI (blue). HCS 1 and HCA identify the sensory patches. RCAS-EGFP expression is expressed throughout the sensory (**A**) and non-sensory regions of the inner ear (**A** and **B**), whereas the RCAN-Hes5-d2EGFP is expressed within the sensory patches only (**C,D,E**). Whole mount ISH using a probe mix of Hes5.1 and Hes5.3 performed on E7 and E10 ears, indicating that Hes5 expression is restricted to sensory patches (**F** and **G**). **E-E'** are higher magnification images of an E10 crista showing that the reporter is active in supporting cells only (**E'**) and not within hair cells (**E**). Scale bar = 100 μm (applies to panel s A, C, D), scale bar = 20 μm (applies to panels B, E, E').

#### **4.1.3 Dynamic changes in fluorescence following Notch inhibition with DAPT**

I have shown that at early stages of ear development, using electroporation of plasmid DNA, the Hes5-d2EGFP reporter is sensitive to an artificial activation of the Notch pathway (see results section 3.2). In order to determine whether the Hes5-d2EGFP reporter could also respond to a reduction of Notch activity, the pharmacological agent DAPT (N-[N-(3, 5-difluorophenacetyl)-1-ala-nyl]-S-phenylglycine t-butyl ester) was used to block Notch signalling in inner ear transfected with RCAN-Hes5-d2EGFP reporter. Treatment with DAPT inhibits gamma-secretase ( $\gamma$ -secretase) activity (Dovey et al., 2001; Geling et al., 2002; Selkoe and Kopan, 2003) and this prevents the S3 cleavage event that releases the active, intracellular domain of Notch receptors, thus preventing the transcription of Notch target genes (Berezovska et al., 2000; De Strooper et al., 1999; Mumm et al., 2000). This mimics a complete loss of canonical Notch activity in vitro and in vivo, regardless of the Notch receptor/ligand pair involved (Geling et al., 2002). Previous studies have shown DAPT to be effective in blocking the Notch pathway in the embryonic chicken retina (Nelson et al., 2006) and inner ear (Abello et al., 2007; Daudet et al., 2007). In cultures of embryonic organ of Corti, DAPT treatment can induce the formation of extra hair cells (Hayashi et al., 2008; Takebayashi et al., 2007), an expected consequence of the loss of lateral inhibition.

To test the effect of DAPT on reporter activity within transfected ears, embryos were electroporated at E2 with either RCAS-EGFP (used as a control) or RCAN-Hes5-d2EGFP then allowed to develop until E8. The inner ears of four RCAS-EGFP and one well-transfected RCAN-Hes5-d2EGFP embryos were then dissected and incubated in the same 35mm culture dish containing DMEM/F12 with 20 $\mu$ M of DAPT. At the onset of the experiment (time 0 mins in Figure 4.4) the RCAS-EGFP samples showed widespread fluorescence throughout the inner ear, while EGFP fluorescence was restricted to the sensory domains in the RCAN-Hes5-d2EGFP specimen. The samples were then maintained in a tissue culture incubator and fluorescence images were collected at 3-hour intervals for 9 hours. The fluorescence of the Hes5-d2EGFP reporter appeared strongly reduced 6 hours after the onset of DAPT treatment and was almost completely absent after 9 hours (see(\*) Figure 4.4), whereas the EGFP fluorescence in the four RCAS-EGFP transfected ears remained constant. This suggested that the Hes5-d2EGFP reporter was indeed sensitive to a reduction in Notch activity induced by DAPT.



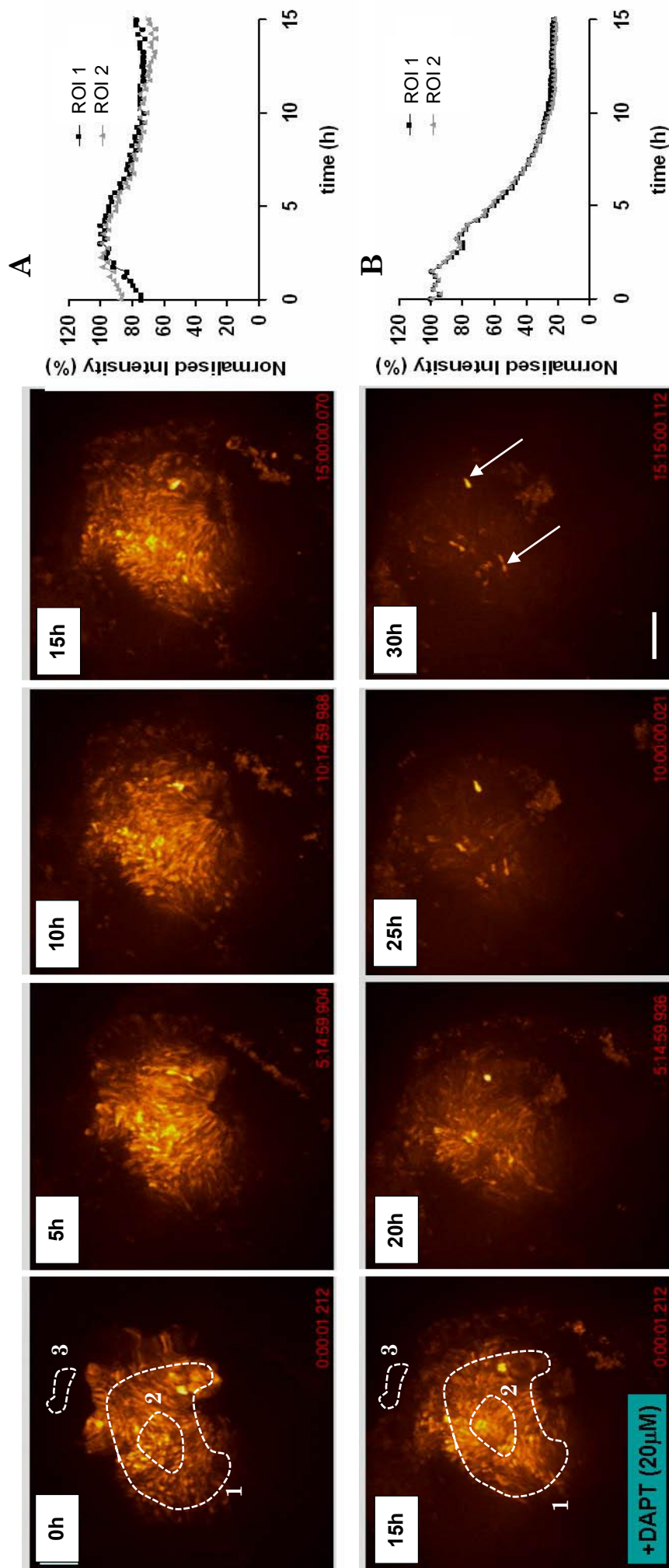
**Figure 4.4 RCAN-Hes5-d2EGFP reporter activity is reduced after DAPT treatment.** Inner ears were electroporated with either RCAS-EGFP or RCANHes5-d2EGFP at E2 and harvested at E8. The inner ears were cultured in DMEM then treated with 20 $\mu$ m DAPT for 9hrs and imaged every three hours. A reduction in EGFP fluorescence is seen in one transfected sensory patch indicated by the asterisk. Scale bar = 50 $\mu$ m. (applies to all panels).

To quantify more accurately the decrease in fluorescence of the reporter consecutive to DAPT treatment, I repeated the experiment using time-lapse imaging on a Nipkow spinning-disc confocal microscope. Fluorescence of the Hes5-d2EGFP reporter is stronger in the sensory crista than in other sensory organs, therefore I focused on these epithelia for analysis. Sensory crista transfected with the RCAN-Hes5-d2EGFP were dissected out of E8 embryos, and maintained in DMEM/F12 medium (without phenol red) into a 35mm glass-bottom Mattek dish. The specimens were immobilized using a platinum harp and incubated at 37°C with CO<sub>2</sub> for 1 hour before imaging. During imaging the dish was kept in a chamber that was heated to 37°C and gased with 5% CO<sub>2</sub>. Confocal z-stacks were collected every 20 minutes. Quantification of the fluorescence levels during the time-lapse experiments was performed as described in 2.6.

In the experiment shown in figure 4.5 (Movie 1), images were taken over a period of 15 hours, during which time the levels of fluorescence fluctuated slightly, but remained relatively high. There was no evidence for photo-bleaching and despite some morphological reorganization of the tissue, dynamic cellular processes such as interkinetic nuclear migration and cell division could be observed and there was little sign of cell death. Following the addition of 20  $\mu$ M DAPT, there was a rapid decrease in fluorescence starting after 2 hours and dropping to a minimum of 20% of its original level after 10 hours. The decrease followed a similar time-course in three separate experiments and the half-life ( $T_{1/2}$ ) of the d2EGFP fluorescence following addition of DAPT was estimated to ~6 hours (n=3). At the end of the experiment, a small proportion of cells remained fluorescent (white arrows in Figure 4.5, time 15h). These cells however exhibited higher fluorescence levels than the other cells within the rest of the epithelium at previous time points. These differences in fluorescence levels could be due to an abnormal response of the Notch reporter, but the detectable decrease in fluorescence within these cells after DAPT addition suggests this is not the case. Another explanation is that levels of Notch activity differ between individual cells, and those with the highest initial levels of Notch activity remain fluorescent for a longer time during DAPT treatment.

The results show that blocking the Notch signalling pathway with DAPT leads to a reduction in fluorescence intensity of the RCAN-Hes5-d2EGFP reporter with a half-life of ~6 hours. Therefore this reporter is sensitive to a reduction in ligand-dependent Notch activity.





**Figure 4.5 Time-lapse imaging of DAPT treated crista transfected with RCAN-Hes5-d2EGFP at E8.** Inner ears were electroporated with RCAN-Hes5-d2EGFP at E8 and a crista was harvested at E8 and cultured in DMEM (control). The crista was first imaged using Nipkow spinning-disc confocal microscope for 15h without DAPT. A confocal z-stack was taken every 20mins. After 15 h, 20 $\mu$ M of DAPT was added and confocal z-stacks were taken every 20mins. The figure shows still images (maximum projection) at 5 h intervals of the original movie. **(A)** Shows the slight fluctuations in fluorescence observed during the imaging, but there is no sign of photo-bleaching. **(B)** Shows that 2 h after the addition of DAPT, there is an 80% reduction in fluorescence and that after 6 h there is a 50% reduction in fluorescence. This movie is representative of three separate experiments that were performed under the same conditions. ROI 1, 2 and 3 (background) are indicated by the white dashed lines. Scale bar = 50 $\mu$ m.

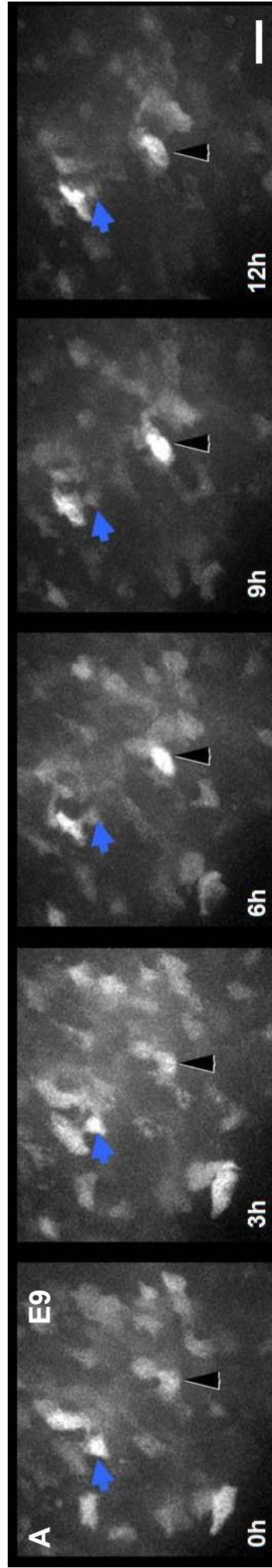
#### ***4.1.4 Dynamic changes in fluorescence can be detected at the cellular level with the RCAN-Hes5-d2EGFP reporter***

To determine whether it is possible using this method to compare the levels of fluorescence in more detail, within individual cells, I imaged a sensory crista transfected with RCAN-Hes5-d2EGFP, at high magnification over a period of 12 hours (see Figure 4.6-Movie 2). The progenitor/supporting cells could be recognized at the surface of the epithelium because of their polygonal shape, and exhibited a range of fluorescence intensities. Some cells were devoid of fluorescence and had a more circular surface, suggesting they could be immature hair cells. This mosaic pattern of activation is similar to the one observed in fixed samples (See Figure 4.10).

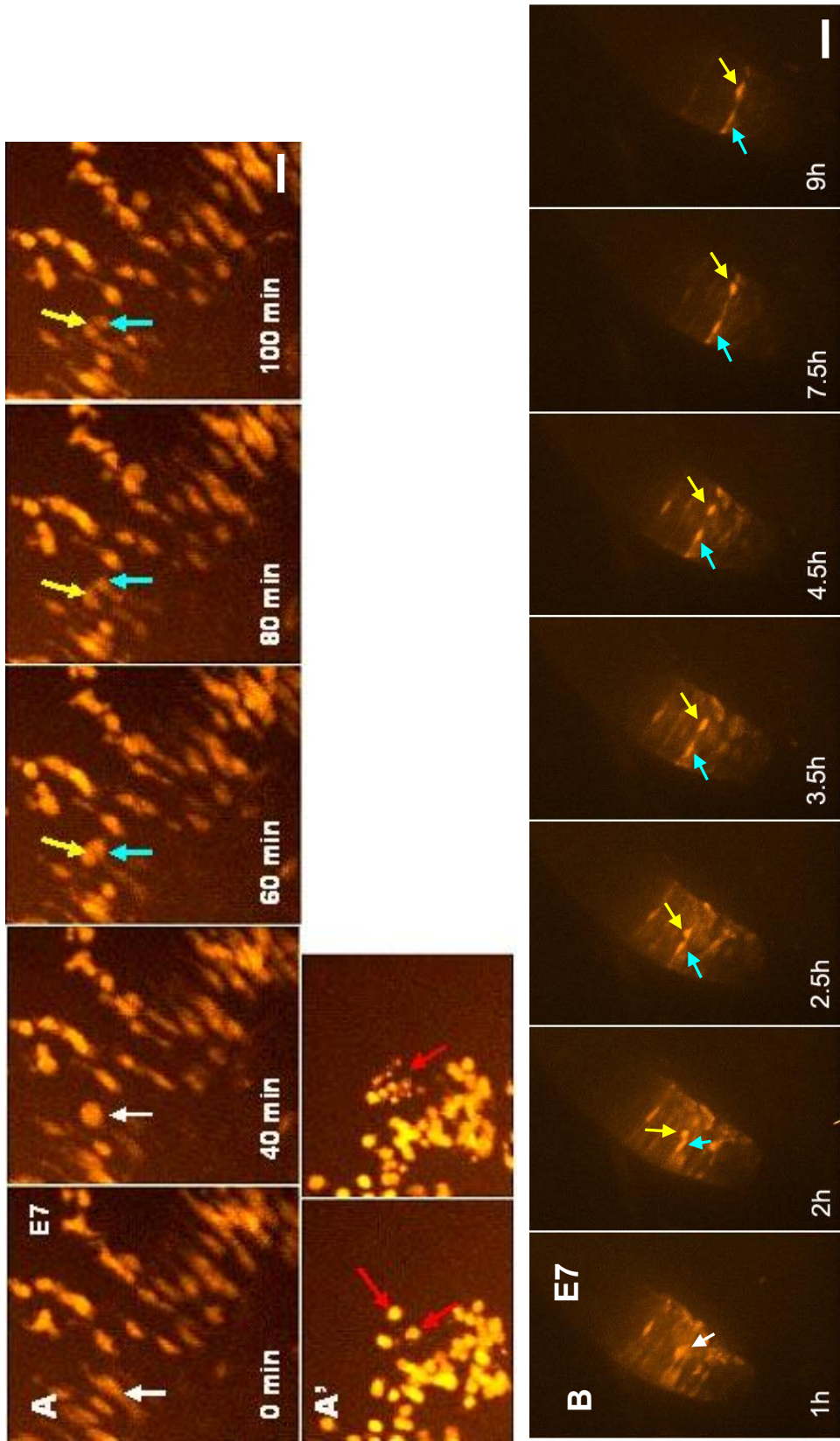
Over the 12 hours in control medium, some of the cells became more fluorescent (black arrowhead in Figure 4.6), while some became less fluorescent (blue arrow in Figure 4.6), most likely reflecting dynamic changes in the levels of Notch activity that the cells were experiencing. However a precise quantification of the signals of individual cells was complicated for several reasons. Firstly, the EGFP is expressed in the cytoplasm that makes it difficult to distinguish adjacent cells; the morphology and volume of the cells also influenced the intensity of the fluorescence. Secondly, even small changes in the position of the sample can modify the focus of the cells and thus the recorded fluorescence intensity. Finally, without an internal control for transfection, it was not possible to determine whether a cell with no fluorescence was not receiving Notch activation, or simply did not contain the Hes5-d2EGFP reporter. Using a 63x lens, even small movements of the tissue could cause significant shifts in the focal optical plane, a complication for the collection and analysis of the fluorescence data.

At 20x magnification, cells were seen migrating through the epithelium whilst undergoing division (Figure 4.7A-Movie 3 and B-Movie 4). Cells which were undergoing division appeared to have the strongest fluorescent levels. For example in Figure 4.7B although there was an overall decrease in intensity levels due to photo-bleaching, the cell that divided appeared to have high levels of Notch activity and so did the resulting daughter cells. Figure 4.7 shows two examples which represent some of the difficulties with time-lapse imaging. Figure 4.7A is an example of cell death occurring when the culturing conditions are not optimal. Figure 4.7B clearly shows there was movement in the X-Y plane and photo-bleaching. Nevertheless, these data indicated that there were clear differences in the levels of fluorescence

of individual cells, and dynamic changes could be detected over time.



**Figure 4.6 Time-lapse imaging of RCAN-Hes5-d2EGFP transfected E9 crista.** Inner ears were electroporated with RCAN-Hes5-d2EGFP at E2 and a transfected crista was harvested and cultured at E9 (A). High magnification (63x) shows changes in reporter activation at the cellular level with the blue arrow showing a cell that appears to be decreasing in fluorescence and the black arrow showing a cell that increases in fluorescence (n = 1). Scale bar = 10  $\mu$ m.



**Figure 4.7 Time-lapse imaging of E7 RCAN-Hes5-42EGFP transfected sensory epithelia. (A)** Transfected basilar papilla (E7). White arrows indicate a cell that undergoes cell division and gives rise to two daughter cells (indicated by the yellow and blue arrows). Within the same sample there is also cell death occurring (**A'**) Two cells are indicated by the red arrows which eventually burst into tiny particles. **(B)** Transfected crista. A section of a time-lapse image sequence which shows cell division occurring. The white arrow indicates a cell that is undergoing division. Photo-bleaching is occurring and movement in the x-y plane. The cells undergoing division appear the brightest. Scale bar in A = 20 μm, B = 100 μm

## **4.2 Tol2 transposon enables stable integration of the Hes5 reporter in the developing chick inner ear**

The experiments with the RCAN-Hes5-d2EGFP showed stable integration of the Hes5 reporter for live-imaging of Notch activity at late stages of inner ear development. There are however some limitations with this approach. Once the tissue or organ of interest is infected by replication-competent virus, new virus particles will constantly be produced, spreading the infection; therefore the time of transgene expression for any given cell is not known. As the infection spreads throughout the sensory organ, the increased number of labelled cells also complicates the tracking of individual cells. Finally, in the absence of an additional fluorescent protein constitutively expressed, it is impossible to determine whether a non-fluorescent cell is Notch-inactive, or has not been infected. To overcome some of these problems and to produce a reporter that could facilitate the study of Notch activity within individual cells, I cloned the Hes5-d2EGFP reporter into a Tol2 transposon vector. The Tol2 vector system (see below for further details) has recently been applied to the chicken system in order to circumvent the problem of transient gene expression when genes are introduced by electroporation (Sato et al., 2007).

The Tol2 system had several advantages for these experiments:

- a) Several Tol2 vectors can be co-electroporated and transfected within cells. Hence a constitutively expressed marker for transfection could be included with the Hes5 reporter.
- b) Transfection with Tol2 is mosaic, which could facilitate analysis at the single cell level.
- c) Tol2 vectors can accommodate much bigger inserts than RCAS vectors. The Tol2 vector can carry a DNA insert as large as 11kb without reducing its transpositional activity (Urasaki et al., 2006). This was particularly important for the following experiments in which the Hes5 element was used to drive Delta1 and EGFP expression (see Chapter 5).

### **4.2.1 Introduction to Transposon elements**

Transposable elements are another powerful tool available for genetic analysis. They can be used as transformation vectors because they can carry a gene or other DNA fragments (Rubin and Spradling, 1982) that can then be used to induce insertion mutations or promoter/enhancer trapping



whereby the element carries a marker gene that lacks a promoter or has a weak promoter (O'Kane and Gehring, 1987). There are two classes of transposon elements: RNA-mediated elements and DNA-based elements (class I and class II elements, respectively). DNA-based elements have been used in bacteria as early as the 1970s and in nematode, *Drosophila* and other eukaryotic organisms during the mid-1980s. They are usually discovered following the structural analysis of mutant genes which results in the identification of extra DNA fragments. With advances in technology, the number of elements discovered in vertebrates rose however most of these were inactive (not carrying a complete internal gene). Elements that had a similar sequence to the *Drosophila* mariner element and the nematode Tc1 element, were found in a range of different organisms and called the mariner/Tc1 family (Plasterk, 1996). Members of the *mariner*/Tc1 family of element, such as *Sleeping beauty*, are being developed by several research groups (Izsvak et al., 2000).

The Tol2 (Transposable element of *Oryzias latipes*, number 2) element was identified from the genome of the medaka fish (*Oryzias latipes*), a small freshwater teleost species native to Asia. The 4.7 kb insertion, was identified from the allele which causes a quasi-albino phenotype (Koga et al., 1996). It was also the first vertebrate DNA-based element shown to be autonomously active.

The sequence of the Tol2 element is similar to those of transposons belonging to the hAT family, namely *hobo*, *Ac* and *Tam* (Koga et al., 1996). Tol2 consists of imperfect terminal inverted repeats (TIR) of 17bp and 19bp and three subterminal repeats of about 30bp located proximal to the TIR. There are also internal inverted repeats of about 300bp. The element contains a gene composed of four exons for its transposase. Koga and Hori (2000) first demonstrated that the gene carried by Tol2 encodes a 649 amino acid protein, a transposase, and that the transposase has entire activity for cut-and-paste transposition. Other groups demonstrated transposition activity in the zebrafish, for excision (Kawakami and Shima, 1999) and insertion (Kawakami et al., 2000). Tol2 is one of the few DNA-based elements so far demonstrated to be active in vertebrates. Tol2 integrates as a single copy and does not cause rearrangement or modification at the target site, except for the creation of an 8 base pair duplication, which appear adjacent to the integrated Tol2 elements (Kawakami et al., 2000).

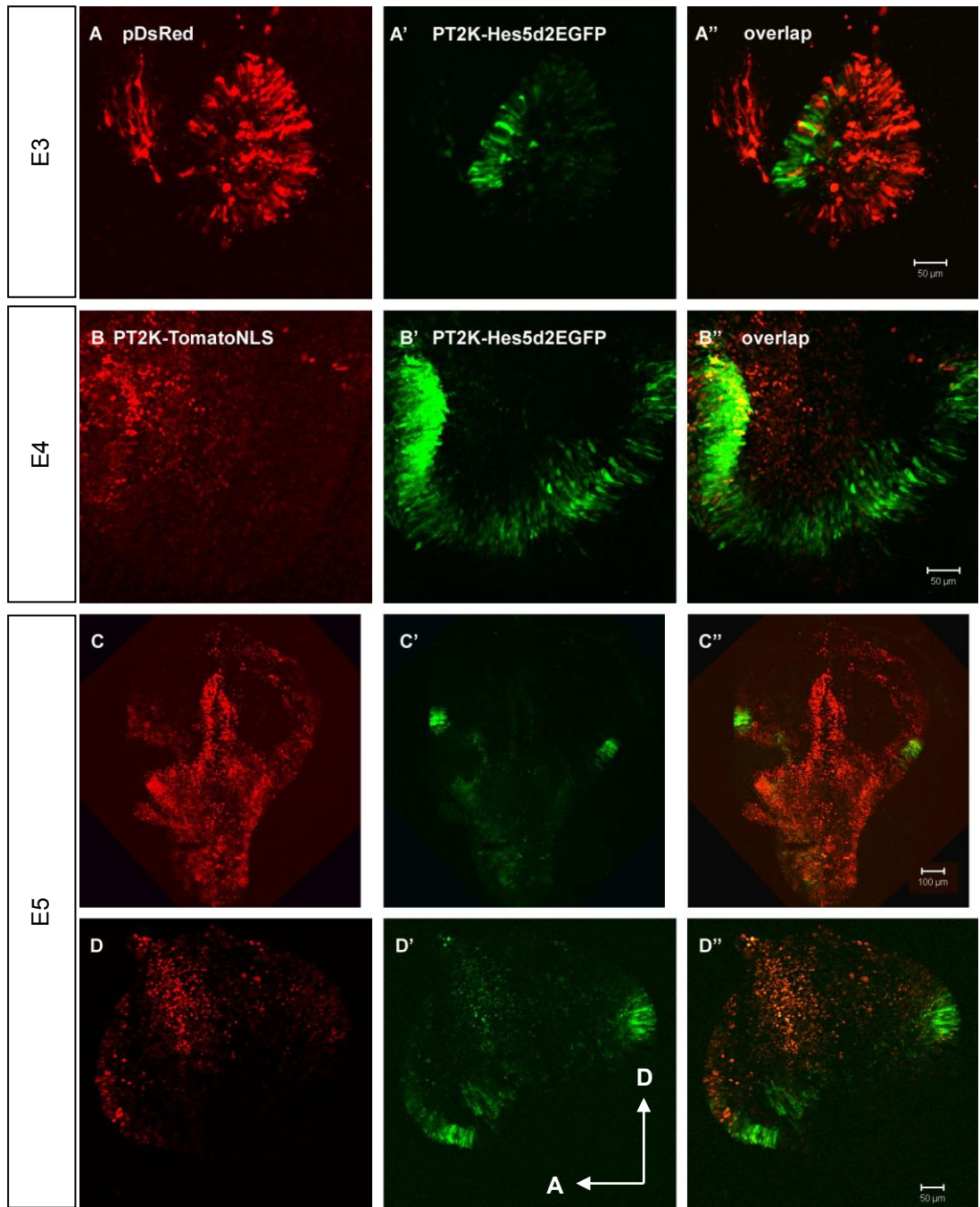
The minimal Tol2 vector, a non-autonomous construct, one has a deletion in the transposase coding region but retains the Tol2 ends, consisting of 200bp and 150bp of DNA from the left and right ends, respectively. Any foreign DNA fragments, up to 11kb, can be cloned between these two sequences (reviewed in Kawakami, 2007; Urasaki et al., 2006). The first study showing that the Tol2 system can be used in chicken embryos to stably integrate an exogenous transgene was by Sato et al., (2007). A plasmid DNA consisting of the Tol2 construct with the GFP expression cassette and a helper plasmid carrying the transposase gene under a ubiquitous promoter (CAGGS) were co-electroporated into the chicken embryo. The Tol2 construct is integrated into the genome soon after electroporation and consequently transfected cells and their progeny that have integrated the transposon express GFP continuously. The expression of GFP was observed up to E8 in somite-derived tissues and E12 in the developing retina (Sato et al., 2007). This study also confirmed chromosomal transposition in chicken, by Southern blot hybridisation. Another study has used this method to elucidate the function of cadherin in dendritic morphogenesis (Tanabe et al., 2006). Therefore the Tol2-mediated gene transfer method is particularly useful for studying the functions of genes during late organogenesis in the chicken embryo.

#### ***4.2.2 Cloning and initial characterisation of the Tol2-version of the Hes5 reporter***

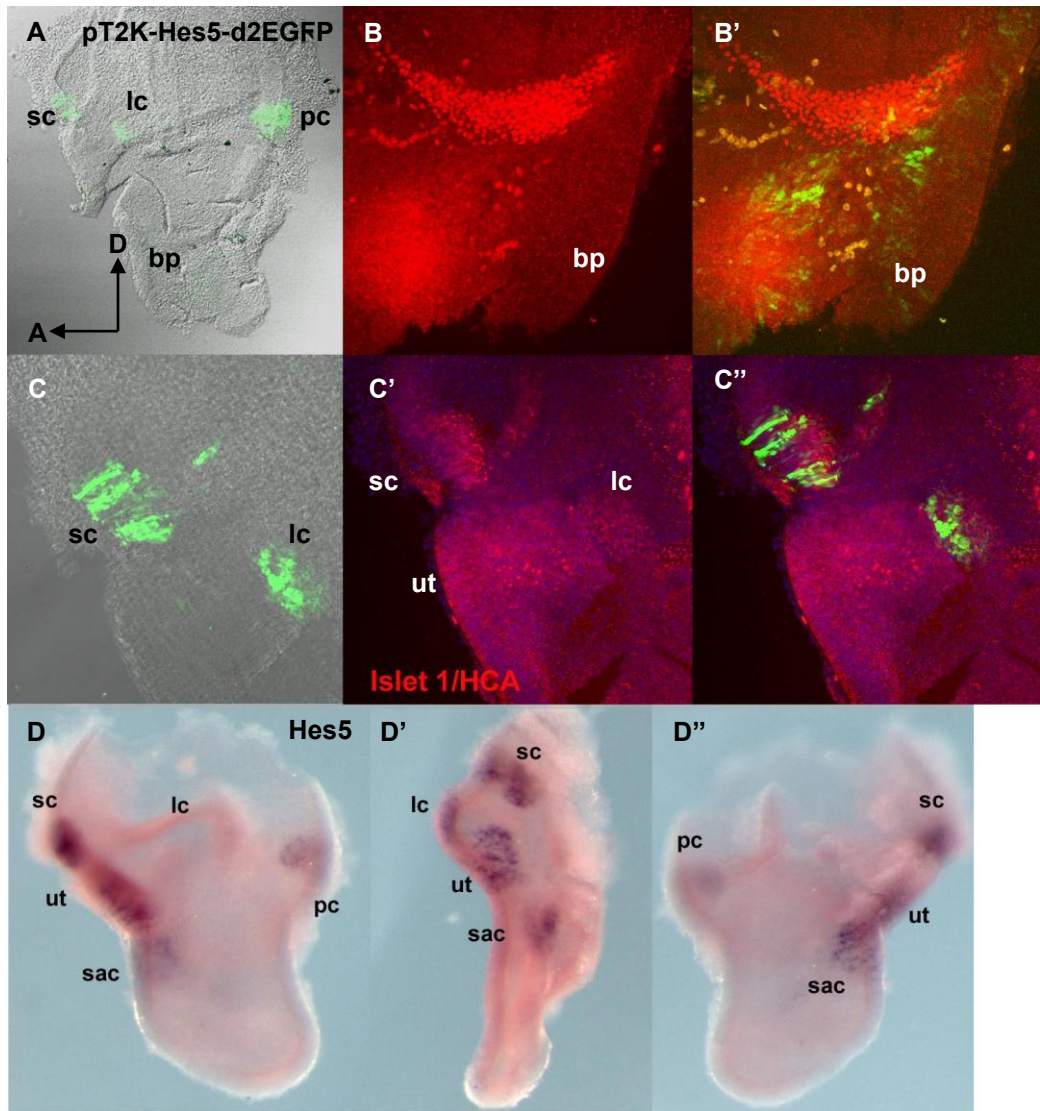
In this study, a Tol2 transposon containing the Hes5 reporter was generated by subcloning the Hes5-d2EGFP cassette into a minimal Tol2 construct (pT2K-CAGGS; see methods 2.2.9). The pT2K-Hes5-d2EGFP, pTPase (encoding the transposase) and the pT2K-TomatoNLS or pDsRed (markers of transfection) plasmids were co-electroporated in the otic cup at E2 and the embryos were returned to the incubator for further development. Twenty-four hours after electroporation, there was strong activation in the anterior domain of the otocyst, a result similar to that obtained with the original pHes5-d2EGFP construct (Figure 4.8A). At E4 there was strong activation in the anterior region that could correspond to the Hes5 patches of expression seen at E4 in the superior crista, utricle and saccule. There was also activation in the posterior region, which corresponds to the Hes5 expression in the posterior crista and in the ventral region, which will form the bp. At E5 the pT2K-Hes5-d2EGFP reporter was strongly activated in the regions of the posterior and superior crista and in the lateral crista (Figure 4.8 and 4.9). There also appeared to be some weak activation in the bp region. At this stage Hes5 is expressed strongly in 5 vestibular patches;



the 3 crista, utricle and saccule (Figure 4.9), however, I did not observe reporter activation in the utricle or saccule in any of the samples at these early stages of inner ear development.

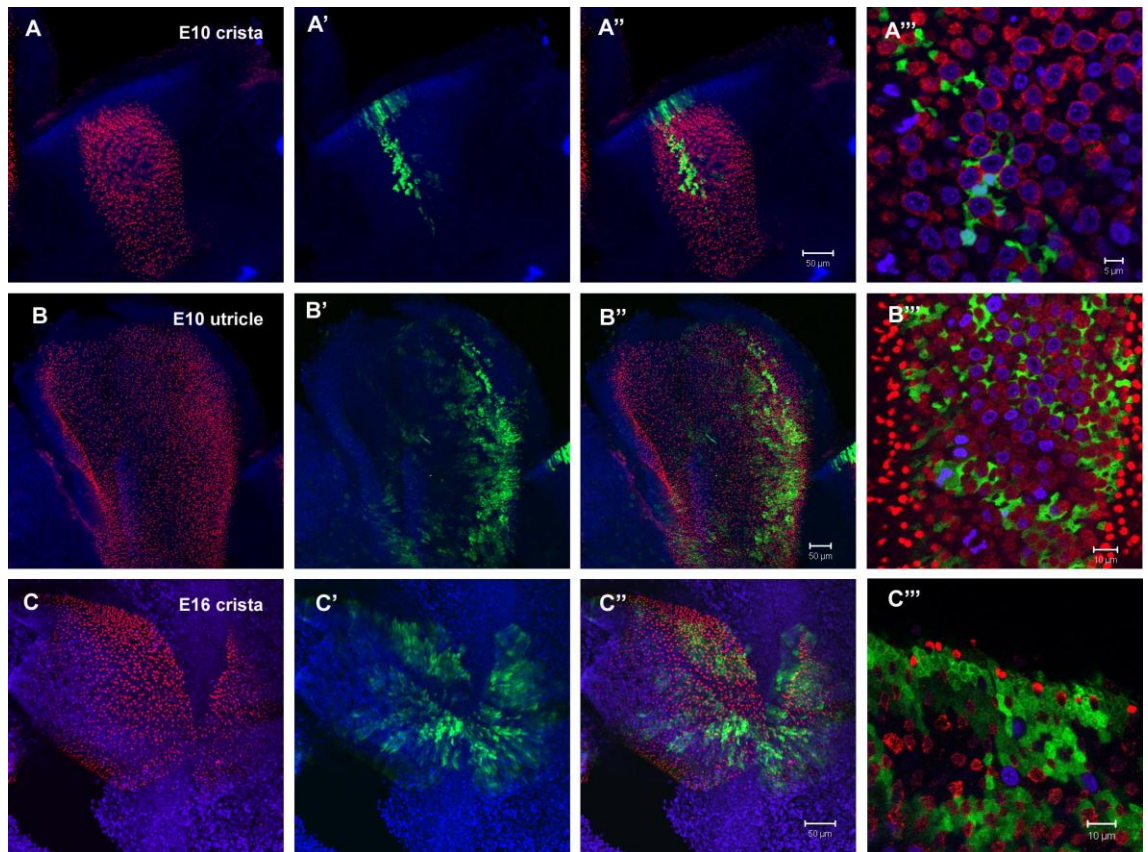


**Figure 4.8 PT2K-Hes5-d2EGFP reporter activation pattern at E3, E4 and E5.** Inner ears were co-electroporated with either pDsRed (**A**) or PT2K-TomatoNLS (**B,C,D**) and pT2K-Hes5-d2EGFP (**A'-D'**) and pTPase at E2 and harvested 24hrs (**A-A''**), 48hrs (**B-B''**) and 72 hrs (**C-D**) later. At E3, activation of the reporter occurs in the anterior region of the otic cup. At E4 there is strong activation in the anterior region which spreads to the posterior region. At E5 the activation pattern of the reporter occurs at distinct regions which will go on to form the superior, lateral and posterior crista. There is a weak activation in the bp region. Scale bar = 50  $\mu$ m in A, B and D and 100  $\mu$ m in C.



**Figure 4.9 PT2K-Hes5-d2EGFP reporter activation pattern at E5. (A)** Bright field whole-mount E5 otocyst. **(B and C)** The pT2K-Hes5-d2EGFP reporter is active within sensory patches as it co-localises with sensory marker islet1 (red) and HCA (red). **(D)** The reporter is active within the regions where Hes5 is expressed. Note Hes5 was detected using a mixture of the Hes5.1 and the Hes5.3 probes, as their expression patterns are the same.

By E10 and E16, and as previously seen with the RCAN-Hes5-d2EGFP, there was stronger activation of the reporter in the cristae than in the utricle, saccule or bp. The pattern of activation matched the endogenous expression pattern of Hes5.1 and Hes5.3 at similar stages. It was also noted that within transfected utricular macula, the activation of the reporter was strongest within the striola region (see figure 4.10 B). It was clear from the high magnification images that the Hes5-driven EGFP fluorescence was restricted to the progenitors/supporting cells of the basal layer of the sensory epithelium (See figure 4.10) and there was no activation of the reporter within hair cells. These results confirm that the Tol2 version of the reporter can induce stable integration of the reporter and that its specific activation pattern within the sensory domains is still preserved. However the pattern of transfection and activity with the PT2K-Hes5-d2EGFP reporter was more mosaic than with the RCAN-Hes5-d2EGFP construct. In most cases small groups of cells and isolated cells were visible, which could facilitate analysis of fluorescence signals and tracking of single cells during live-cell imaging.



**Figure 4.10 PT2K-Hes5-d2EGFP activation patterns.** Sensory patches, crista and utricle, are indicated by the combined hair cell markers (HCA and HCS1 in red) (**A-C**). Activation of the PT2K-Hes5-d2EGFP reporter within the sensory crista and utricle (**A'-C'**). (**A'''-C'''**) are high magnification images of region of transfection which indicates that activation is not within hair cells, but in supporting cells/progenitor cells only. DAPI staining of the nucleus in blue. NOTE: the focal plane in **C'''** is at the apical surface where there are few nuclei.



#### **4.2.3 Blocking Notch signalling with DAPT decreases fluorescence from the PT2K-Hes5-d2EGFP reporter**

In order to confirm that the PT2K-Hes5-d2EGFP reporter is also sensitive to variations of Notch activity, I repeated the experiments using DAPT to block Notch signalling and analysed the resulting changes in fluorescence signals with time-lapse confocal microscopy. Embryos transfected with PT2K-Hes5-d2EGFP and PT2K-TomatoNLS were allowed to develop until E10-E13. Inner ears were dissected under sterile conditions and checked for fluorescence. In order to minimise the problem of tissue movement that occurred with the platinum harp, a different method was applied to immobilise the tissue. Transfected inner ear sensory patches were immobilised using Cell-Tak cell and tissue adhesive in MatTek glass-bottomed dishes and cultured for 24 hours before DAPT treatment and imaging. The culture medium was then removed and replaced with DMEM containing 20  $\mu$ M DAPT. For red and green dual fluorescence imaging, the LSM 510 Zeiss inverted confocal microscope was used instead of the spinning disc, which lacked the appropriate filters. The laser powers were kept to a minimum and z-stacks were collected every 20 minutes only to avoid photo-bleaching.

In the experiment shown in figure 4.11 (Movie 5), there was a rapid decrease in EGFP fluorescence starting approximately 3 hours after the addition of DAPT. It took approximately 7 hours for the EGFP fluorescence to reach 50% of its maximum intensity. The nuclear tomato fluorescence remained stable throughout the experiment. The decrease in EGFP fluorescence followed a similar time-course and profile to that observed with the RCAN-Hes5-d2EGFP. Three separate experiments were performed under the same conditions. On each occasion the reporter activity decreased with similar kinetics (see figure 4.12).

The use of Cell-Tak was found to greatly reduce movements of the tissue during imaging, however successful adhesion of the tissue was variable, limiting the number of samples that could be successfully imaged for long periods of time. There appeared to be very little photo-bleaching as the level of fluorescence from the control plasmid, PT2K-TomatoNLS, remained high as the EGFP decreased and there was little sign of cell death. The advantage of using the PT2K-TomatoNLS as a control is that cells that have been transfected can be identified. In the region of interest in figure 4.12, cells which were transfected with the nuclear-tomato, but are not EGFP

positive, potentially have down regulated Notch activity and differentiated into hair cells.

#### ***4.2.4 Do the changes in Hes5 reporter fluorescence mimic the changes in endogenous Hes5 expression after DAPT treatment?***

There is nothing known about the dynamics of Notch activity during hair cell fate decisions in the inner ear. It has been shown, using a Hes1 luciferase reporter in transgenic mice, that during somitogenesis, expression of Hes1 oscillates (Masamizu et al., 2006). It has also been suggested that oscillations of Notch activity and proneural gene activity could occur during neurogenesis in vertebrates (Kageyama et al., 2008). Similar oscillations might occur in inner ear progenitor cells, but could the Hes5 reporter be used to investigate these?

The following experiments were performed by Sophia Hafner in order to test how changes in fluorescence of the Hes5 reporter, that I have measured, relate to changes in endogenous levels of Hes5 gene expression. Hes5.1 mRNA levels were measured by quantitative Real-time PCR in organotypic cultures of E10 inner ear epithelia treated with 20  $\mu$ M DAPT and sampled at different times over a 7.5 h period. I have normalised the mRNA data (from Sophia Hafner) and the GFP (protein) fluorescence data in figure 4.12. The results indicated that there is ~2.5 hours delay in the decrease of the Hes5-d2EGFP fluorescence levels following DAPT treatment when compared to the decrease in Hes5.1 mRNA levels. This delay is expected and represents the time it takes for the degradation of EGFP mRNA and protein. These data indicate that with the present Hes5 reporter, it would not be possible to monitor changes occurring over minutes to a few hours or oscillations in the levels of endogenous Notch activity. However this reporter could be useful to analyse long-term and unidirectional changes in Notch activity, such as those that may occur during commitment of progenitor cells to a hair cell fate.

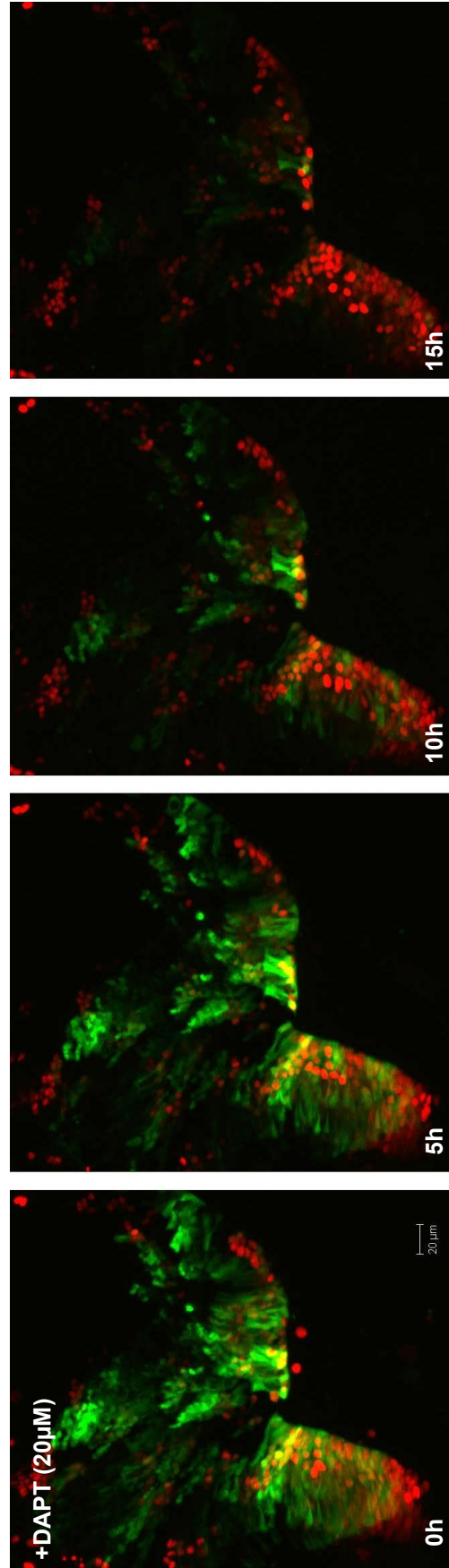
#### ***4.2.5 Live imaging of embryonic sensory epithelia reveals extensive proliferation at the time of hair cell formation***

Dynamic processes such as interkinetic nuclear migration and cell division of sensory progenitor cells could be observed during time-lapse movies. Figure 4.13 shows an image series from a region of interest taken from within the transfected region of the crista shown in figure 4.11. This region clearly shows how the Hes5-d2EGFP reporter can be used to visualise the morphology of single cells during cell division and that the PT2K-TomatoNLS can be used to follow the movements of the nucleus. The nuclei of dividing cells travel up towards the apical surface of the epithelium, where division occurs, then the two daughter nuclei move back down

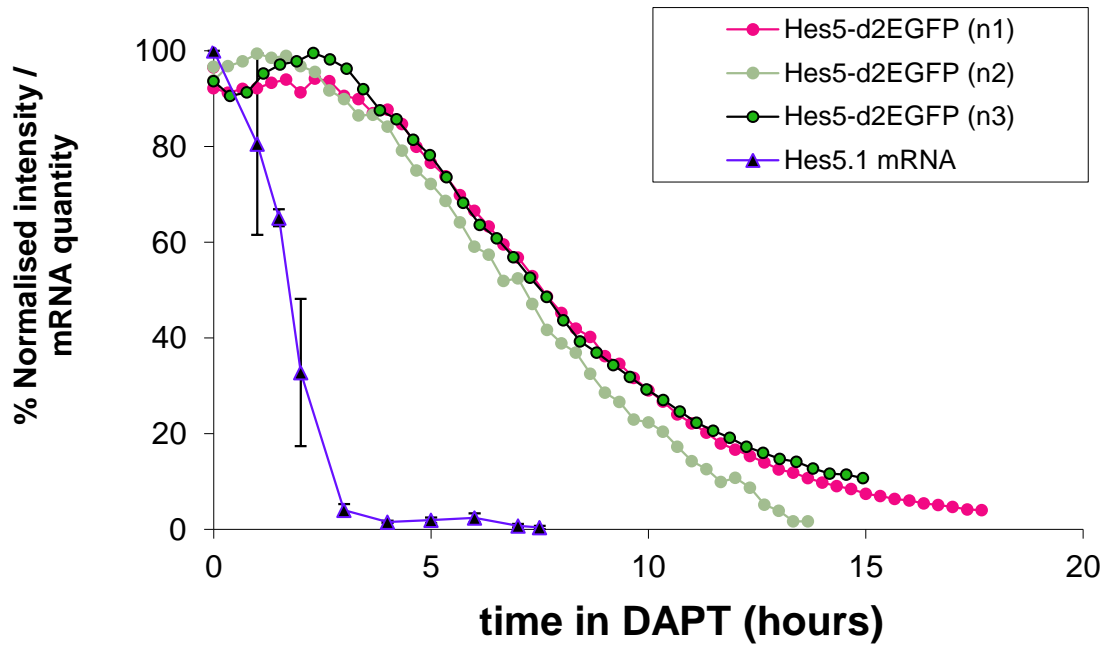


towards the basal plane of the epithelium. It is estimated that the duration of mitosis, that is from the onset of apical migration to the completion of anaphase is ~1-2 hours. After mitosis, daughter cells retained a progenitor-like morphology but determining the ultimate fate of these cells requires longer time-lapse experiments. Hair cells (with an apical nucleus and no or very low GFP fluorescence) were seen in proximity to dividing cells, indicating that there is some overlap between hair cell differentiation and progenitor cell proliferation in these specimens.

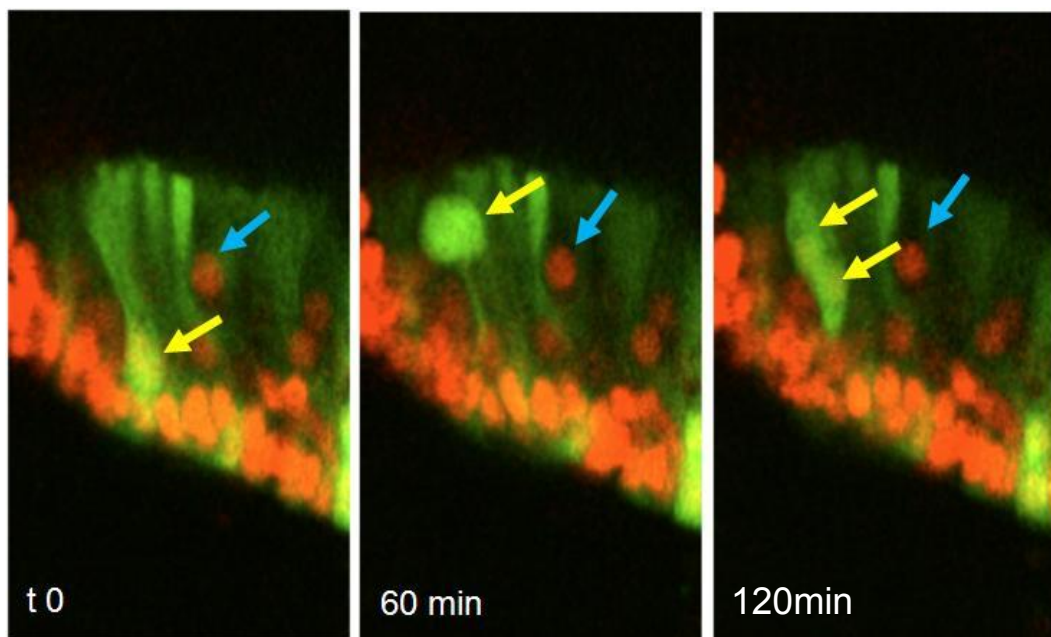
In summary, the Hes5-d2EGFP reporter can be stably integrated into the developing chicken inner ear using either RCAN retrovirus or Tol2 transposon. Experiments with DAPT confirmed that the Hes5 reporter is sensitive to canonical, gamma-secretase dependent Notch signalling. The co-electroporation of PT2K-Hes5-d2EGFP reporter with other Tol2 constructs such as PT2K-TomatoNLS greatly facilitated the identification of the cells that had downregulated Notch activity.



**Figure 4.11 Time-lapse of DAPT treated crista transfected with PT2K-Hes5-d2EGFP and pT2K-TomatoNLS at E10.** Inner ears were electroporated with PT2K-Hes5-d2EGFP, PT2K-TomatoNLS and transposase at E2 and a crista was harvested at E10 and cultured in DMEM for 24h. After 24h the solution was replaced with DMEM+20µM DAPT and confocal z-stack was taken every 20mins. The figure shows still images (maximum projection) at 5 h intervals of the original movie. 2-3 h after the addition of DAPT, there is a reduction of EGFP fluorescence, with a half-life of approximately 7 h. The nuclear Tomato fluorescence remains stable over time. This movie is representative of 3 separate experiments that were performed under the same conditions.



**Figure 4.13 Time-course of EGFP fluorescence and Hes5.1 mRNA following DAPT treatment.** The otic cups were transfected with PT2K-Hes5-d2EGFP (n1 and n2) and PT2K-TomatoNLS. Transfected sensory epithelia were harvested at E10-E13 and cultured in DMEM/F12 for 24 h before imaging. Following 20  $\mu$ M DAPT treatment, confocal z-stacks were collected every 20 minutes. The fluorescent intensity was measured in two regions of interest (ROI) within each transfected epithelia. The background levels were subtracted from each ROI, and the values were normalised to the maximum intensity. The average of the two ROI were then plotted (filled circles). Hes5.1 mRNA levels were measured, by qRT-PCR, in organotypic cultures of E10 inner ear epithelia treated for 7.5 h with 20  $\mu$ M DAPT (n=3 independent series) (filled purple triangles). The time-course of the EGFP decrease following DAPT treatment for the two versions of the reporter, RCAN-Hes5-d2EGFP (n3) and the PT2K-Hes5-d2EGFP (n1 and n2) are similar.



**Figure 4.12 Interkinetic nuclear migrations.** This is a selected region of interest from the figure 4.11 which shows that it is possible to observe interkinetic nuclear migration during cell division (yellow arrows). In this case the daughter cells have notch activity. Cells which are transfected with the nuclear-tomato, but are not EGFP positive can be observed. It is possible that these cells have down regulated Notch activity and differentiated into hair cells (blue arrows).

# 5

## ROLES OF LATERAL INDUCTION AND LATERAL INHIBITION: INSIGHTS FROM A DELTA1 GAIN-OF-FUNCTION STUDY

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Lateral induction is a mechanism whereby a cell expressing a DSL ligand activates Notch and expression of a DSL ligand in the neighbouring cells, thus generating an intercellular positive-feedback loop. This cooperation results in a uniformly high level of ligand expression in groups of interacting cells. There is mounting evidence that an early phase of Notch activity, dependent on lateral induction and the ligand Serrate1 is important for the normal development of sensory patches (Daudet et al., 2007; Daudet and Lewis, 2005; Eddison et al., 2000; Hartman et al., 2010; Neves et al., 2011; Pan et al., 2010; Yamamoto et al., 2011). However, the exact roles of lateral induction during sensory patch formation are still unclear. It is not known whether it functions to promote expansion of prosensory patches or if it is required for the formation of boundaries between sensory and non-sensory regions. Previous studies have focused on loss-of-function studies and there has been by comparison a lack of gain-of-function studies. The aim of this study was to test the function of lateral induction using a gain-of-function approach.

I used the Hes5 promoter region previously characterised in the reporter study to drive expression of the Notch ligand Delta1 in the developing chick inner ear. By doing so, the levels of expression of Notch ligands were increased in Notch active cells. The data show that this artificial 'gain of lateral induction' can induce a range of morphological defects of the inner

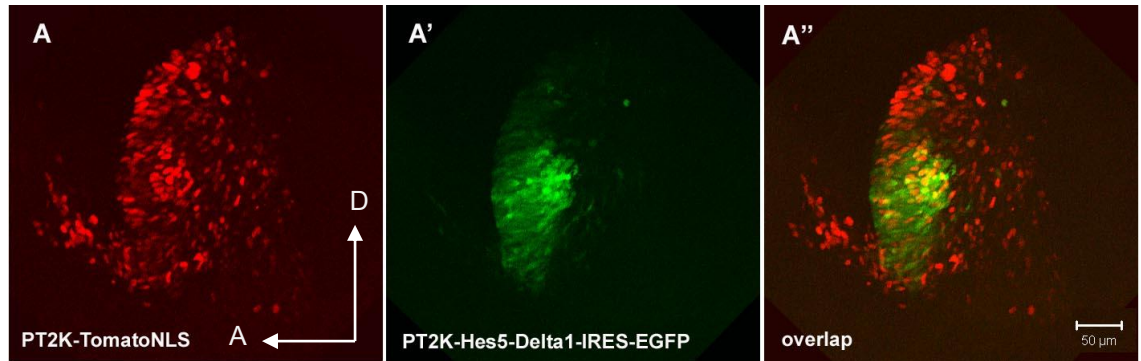
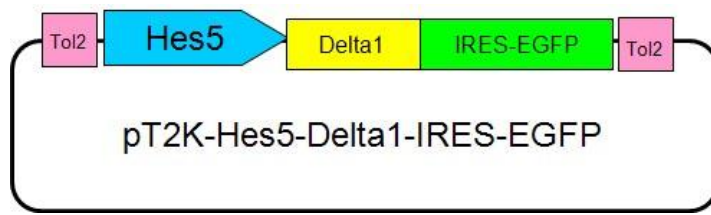
ear, affecting drastically sensory patch size and boundaries. Although the initial aim was to investigate the role of lateral induction in the development of the prosensory domains, I also observed striking effects on hair cell formation which provide new insights into the functions of Delta1 during lateral inhibition.

## **5.1 Cloning and validation of the pT2K-Hes5-Delta1-IRES-EGFP construct**

### ***5.1.1 The pT2K-Hes5-Delta1-IRES-EGFP construct is sensitive to endogenous Notch activity***

The Hes5 promoter has been previously characterised using a green fluorescent protein as a reporter (Chapters 3 and 4) but it can be used to regulate expression of any gene of interest in a 'Notch-responsive' manner. In this study, I used the Hes5 promoter to control the expression of Delta1 along with a stable form of EGFP to identify unambiguously transfected cells. The cloning was done in two steps. First I inserted the 0.7kb Hes5 cis-regulatory element upstream of a Delta1-IRES-EGFP coding sequence, contained in a pre-existing vector (pCAS-Delta1-IRES-EGFP; a kind gift of Domingos Henrique). The Hes5-Delta1-IRES-EGFP cassette (4.4kb) was then cloned by PCR into a promoter-less version of the Tol2 transposon (see Methods for further details).

In order to see whether the pT2K-Hes5-Delta1-IRES-EGFP construct is sensitive to Notch activity, the plasmid was co-electroporated with the pTPase and pT2K-TomatoNLS, as a marker of transfection, in the otic cup at E2. Twenty-four hours after electroporation, the eggs were windowed and checked for EGFP fluorescence using a high-resolution fluorescence stereomicroscope or imaged using confocal microscopy. EGFP was expressed in the anterior neurogenic region where there is endogenous Notch activity (Figure 5.1). This pattern of EGFP expression is the same as the one previously observed with the Hes5-d2EGFP reporter at this stage (See figure 3.3 panel B').

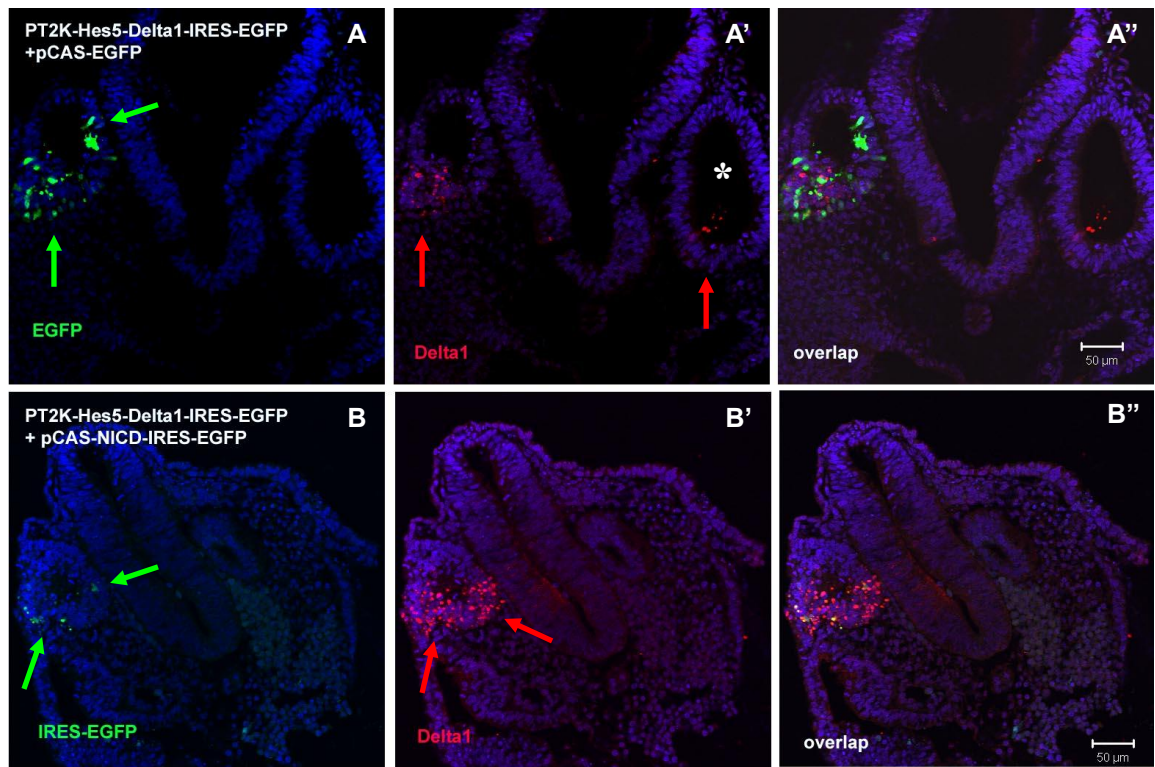


**Figure 5.1 PT2K-Hes5-Delta1-IRES-EGFP activation pattern at E3.** Inner ears were co-electroporated with PT2K-TomatoNLS (**A**) and PT2K-Hes5-Delta1-IRES-EGFP (**A'**) at E2 and harvested after 24hrs. Activation of this construct occurs in the anterior region of the otic cup, where Notch is active (**A''**). The activation pattern is the same as that of the pHes5-d2EGFP reporter (see figure 3.3 panel B').

### **5.1.2 *PT2K-Hes5-Delta1-IRES-EGFP drives Delta1 expression in Notch-active cells***

I expected that cells transfected with this construct would upregulate Delta1 expression in response to Notch activity. To test this, the construct was electroporated at E2 and the embryos were incubated for a further 24 hours, then fixed and immunostained for Delta1 protein at E3. The expression level of Delta1 was compared to the endogenous Delta1 expression pattern in the non-electroporated ear (see Figure 5.2 A'). The results of this experiment showed that there was an increase in expression of Delta1 in the neurogenic domain of the electroporated otocyst compared to the endogenous level of Delta1 expression (n = 3/3). However, there was no ectopic expression of Delta1 in transfected otocyst: elevated levels of Delta1 were only found in the anterior, neurogenic region.

I next tested whether the pT2K-Hes5-Delta1-IRES-EGFP construct would respond to an artificial increase in Notch activity. I co-electroporated pNICD-IRES-EGFP with the pT2K-Hes5-Delta1-IRES-EGFP at E2. The embryos were then harvested at E3, fixed and sectioned for immunostaining with the anti-Delta1 antibody. In the co-electroporated otocyst, there was a strong, widespread expression of Delta1. However Delta1 expression was not restricted to the neurogenic domain but was expressed in the regions where there was ectopic Notch activation as well as in the neurogenic domain where endogenous Notch activation occurs (see Figure 5.2 B'). Although the IRES-GFP fluorescence was weak, it was clear that there was a stronger and more widespread Delta1 expression when the otic cup was co-transfected with pNICD-IRES-EGFP (n = 3/3). This data suggests that the construct is sensitive to Notch activity and that it efficiently induces Delta1 expression within Notch-active cells.



**Figure 5.2 PT2K-Hes5-Delta1-IRES-EGFP drives Delta1 expression in Notch-active cells.** PT2K-Hes5-Delta1-IRES-EGFP was co-electroporated with either pCAS-EGFP (control) or pCAS-NICD-IRES-EGFP at E2. Embryos were harvested and sectioned at E3 then stained with anti-Delta1(red). **(A-A'')** PT2K-Hes5-Delta1-IRES-EGFP induces strong Delta1 expression compared to the control, non-electroporated ear (\*), and expression is restricted to the anterior region. (red arrows). **(B-B'')** Ectopic Notch activity leads to ectopic Delta1 expression (n = 3). Scale bars, 50μm.

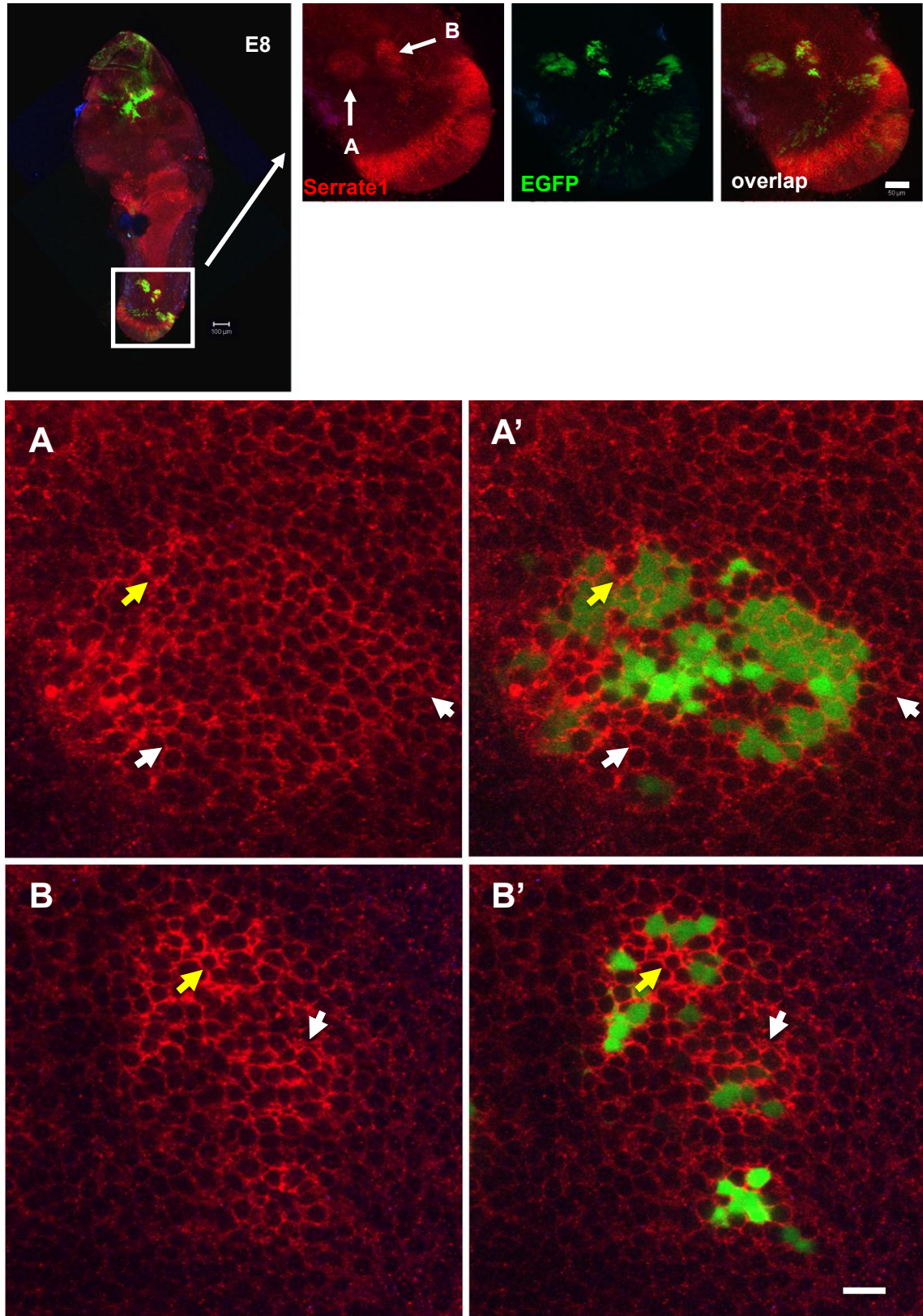


### **5.1.3 *Hes5-Delta1 induces Serrate1 expression***

Serrate1 is thought to be the main ligand associated with Notch-mediated lateral induction in the embryonic inner ear. However, for this experiment, I used Delta1 to artificially induce a 'gain-of-lateral induction'. There were several reasons for selecting Delta1 rather than Serrate1 in these experiments. Firstly, marked elevation of Notch1-ICD levels in the mouse inner ear at the time of hair cell formation suggests that Delta1 may induce Notch activity more strongly than Serrate1 (Murata 2006). Secondly, ectopic expression of Delta1 would be easier to check in sensory progenitor cells than that of Serrate1, which is normally expressed in these cells. Finally, these experiments might reveal whether there is any DSL ligand specificity in the prosensory function of lateral induction.

In order to test the ability of Delta1 at mimicking Serrate1-dependent lateral induction, I investigated whether the pT2K-Hes5-Delta1-IRES-EGFP construct could induce the expression of Serrate1. Serrate1 expression was analysed by immunostaining within E8 pT2K-Hes5-Delta1-IRES-EGFP transfected ears. EGFP fluorescence was detected within sensory domains of the inner ears transfected with pT2K-Hes5-Delta1-IRES-EGFP as expected. This indicated that the Hes5-Delta1-IRES-GFP cassette was stably integrated into the genome and therefore EGFP fluorescence marked the cells that had been transfected and thus expressed Delta1. It was also noted that the inner ears appeared to have some morphological defects (see figure 5.3 and the following results section 5.2.1).

In transfected regions there was an increased level of Serrate1 expression within cells directly adjacent to EGFP positive cells (in trans) and within cells surrounding the EGFP positive regions (See transfected regions highlighted in figure 5.3 A and B). This was observed in three different samples in two separate experiments. These results show that overexpression of Delta1 induces Serrate1 in trans, thus verifying that the construct enhances lateral induction. In addition, they suggest that lateral induction, whereby Serrate1 is positively regulated by Notch, may not be ligand specific.



**Figure 5.3 PT2K-Hes5-Delta1-IRES-EGFP induces *Serrate1* expression.** E8 transfected inner ear has morphological defects which are described in section 5.2.1. A higher magnification view of the basilar papilla shows that there is higher *Serrate1* (red) expression within two transfected regions (EGFP), A and B (indicated by white arrows). The two regions, A and B, with induced *Serrate1* expression are shown at higher magnification. Cells directly adjacent to (yellow arrows) and surrounding (white arrowheads) the transfected EGFP positive cells have a higher level of *Serrate1* expression ( $n = 3$  in 2 separate experiments). Scale bar = 50µm and 10µm for panels A and B.



## 5.2 Transfection with Hes5-Delta1 affects inner ear morphology

### 5.2.1 *Transfection with Hes5-Delta1 within the sensory epithelium altered the gross morphology of the inner ear*

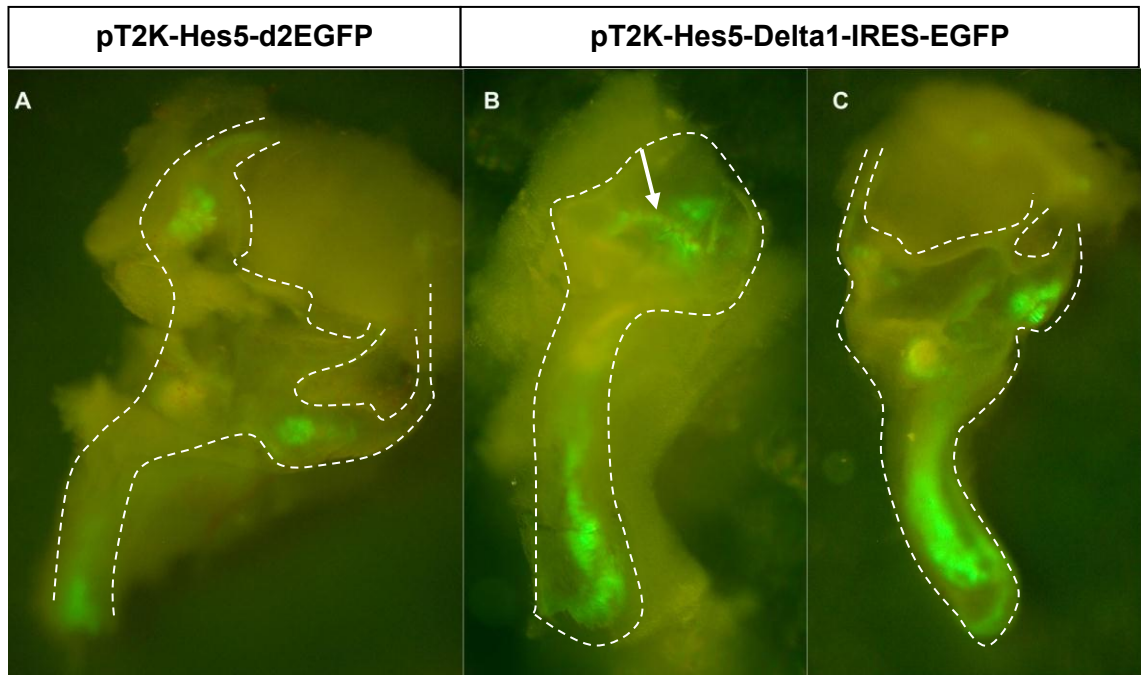
To study the consequences of an artificial enhancement of lateral induction on sensory patch formation, the pT2K-Hes5-Delta1-IRES-EGFP construct was electroporated into the E2 otic cup and the inner ears were collected at various stages ranging from E7 to E14. Immunostaining of hair cells, with anti-HCA/HCS1, and sensory regions, with either anti-Prox1 or anti-Serrate1 on whole-mounts were used to examine the sensory organ distribution and size of sensory patches. Initial experiments included serial sections through transfected inner ears (see Appendix), however, to get a clearer idea of the morphological defects, inner ear whole-mounts at various stages were used for the analysis.

All except one of the pT2K-Hes5-Delta1-IRES-EGFP experimental ears (analysed at E7, E8, E9, E11 and E14) showed gross morphological defects in the vestibular region, where the vestibular region was smaller than that of the contralateral ear (38 out of 39). However the phenotypes were variable and I scored these according to criteria in Table 5.1.

Inner ear phenotypes that were classified as severe (n = 31 out of 38), had a small vestibular region, absent or retarded development of semicircular canals and cochlear duct and/or fewer than five distinct and identifiable sensory patches (for example see figure 5.4B and 5.5B-D and F). For comparison, I used both untransfected contralateral ears and inner ears transfected with pT2K-Hes5-d2EGFP, in which at these developmental stages all sensory patches are present (see figure 5.4A and 5.5A). In general, the anatomy of the pT2K-Hes5-Delta1-IRES-EGFP transfected ears suggested that they had been arrested at an earlier stage of development (Figure 5.5B-D). In some cases with severe abnormalities in the inner ear morphology, there was reduced or absent GFP expression, which could be found mainly in crista-like sensory patches (see figure 5.5B-D n = 16 out of 31). In figure 5.5 there are three examples of E8 ears and one example of an E11 ear that would be described as having severe abnormalities (B-D and F) with some sensory patches present and low levels of EGFP fluorescence. In

addition 15 out of 31 experimental ears showed cochlea abnormalities; the cochlear duct was either shorter than in control ears or totally absent (see figure 5.5). This low GFP may be accounted for by either a lack of transposase being transfected, cell death, or the cessation of transcription from the Hes5 promoter.

In the remaining cases (7 out of 38), the inner ears had milder defects that occurred mainly in the vestibular region and did not affect cochlear development. No more than one vestibular patch was missing or could not be identified. In all cases the size of the vestibular region was smaller than non-electroporated or control ears transfected with pT2K-Hes5-d2EGFP. In these cases where the phenotype was milder there was strong expression of EGFP restricted to distinct sensory patches (for example see figure 5.4C).



**Figure 5.4 The effects of PT2K-Hes5-Delta1-IRES-EGFP on inner ear morphogenesis.** Inner ears were electroporated at E2 with either pT2K-Hes5-d2EGFP or pT2K-Hes5-Delta1-IRES-EGFP and allowed to develop to E8. EGFP is detected within sensory regions only (**A**). E8 inner ears transfected with pT2K-Hes5-d2EGFP has no morphological abnormalities. (**B and C**) Experimental ears transfected with the PT2K-Hes5-Delta1-IRES-EGFP construct shows signs of gross morphological abnormalities. These have a smaller vestibular region. In one case there is a band of EGFP expression across the vestibule where crista appear to be fused (white arrow) and expression in distinct sensory organs cannot be identified (**B**). In some cases there is a smaller vestibular region, but activation within distinct sensory organs can still be identified (**C**). The basilar papilla appears normal in these cases. **B** and **C** are examples of inner ears classified as severe (s) and mild (m), respectively.

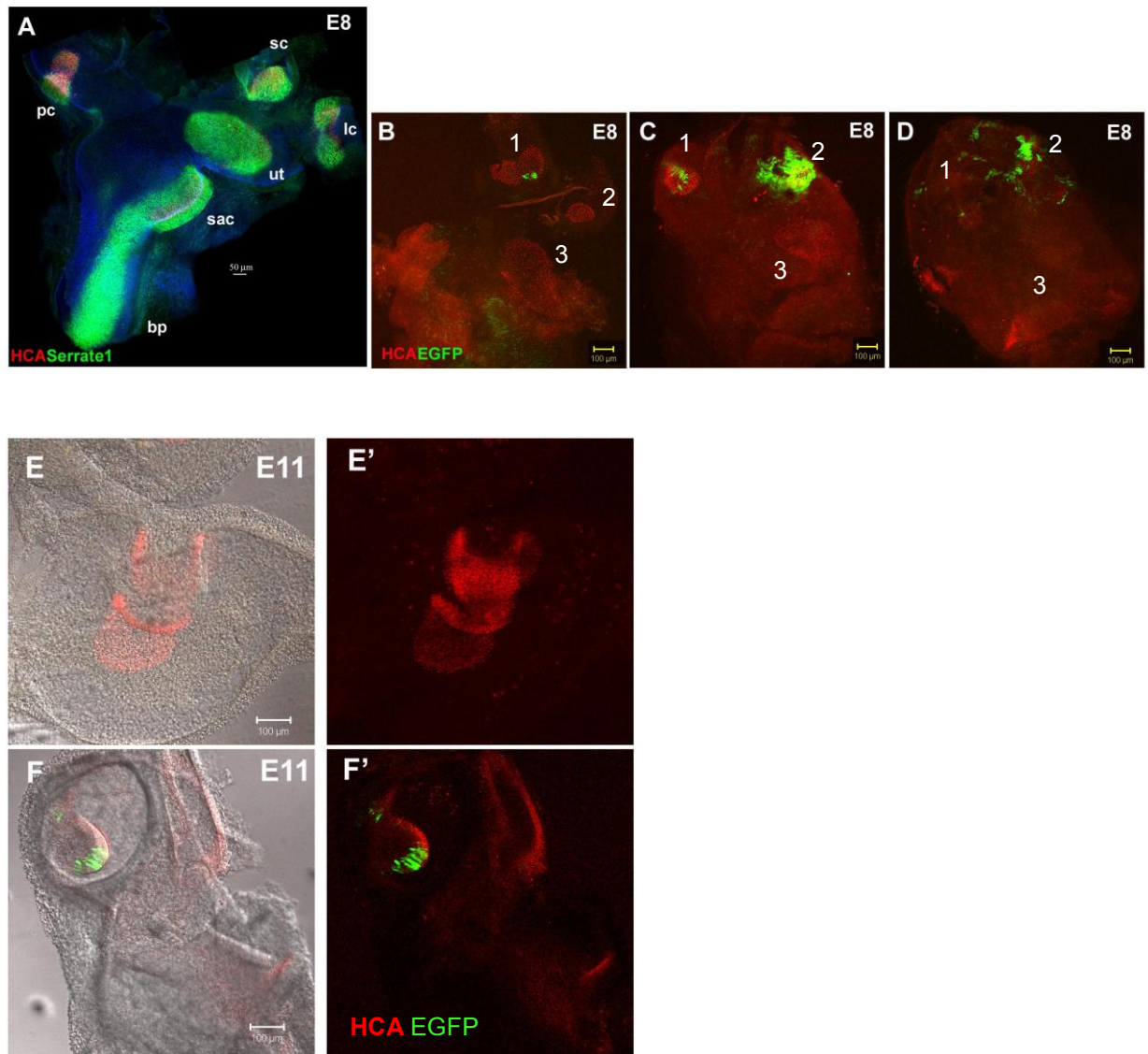


Stage	EGFP fluorescence levels	Number of patches	vestibular region	cochlear duct	Severe (s)/mild(m) phenotype	Fusion of vestibular patches	Total number
E7	low	4	small	absent	s	no	1
	high	*	small	normal	s	yes	6
E8	low	4	small	absent	s	no	1
	low	4	small	absent	s	no	1
	low	1	small	absent	s	no	1
	low	4	small	absent	s	no	1
	high	6	small	normal	m	no	1
	high	6	small	normal	m	no	1
	low	5	small	absent	s	no	1
	no GFP	*	small	absent	s	no	1
	low	5	small	absent	s	no	1
	high	5	small	normal	m	no	1
	low	5	small	normal	m	no	1
	low	6	normal	normal	m	no	1
	high	*	small	small	s	yes	1
	high	*	small	small	s	yes	1
E9	low	5	small	absent	s	no	1
	low	2	small	normal	s	no	1
	low	3	small	normal	s	no	1
	high	5	small	absent	s	no	1
	low	1	small	normal	s	no	1
E11	low	1	small	absent	s	no	1
	low	1	small	absent	s	no	1
	low	5	small	absent	s	no	1
	low	2	small	present	s	no	1
	high	6	small	present	m	no	1
	high	6	small	present	m	no	1
	high	6	small	present	m	no	1
E14	high	*	small	present	s	yes	6

**Table 5.1 Morphological observations of pHes5-Delta1-IRES-EGFP transfected inner ears.** The EGFP fluorescence levels were classed as low if there was very little EGFP fluorescence overall and present only in 1 or 2 sensory patches (in general corresponding to the crista). The EGFP fluorescence levels were classed as high if there was widespread expression throughout all or most of the sensory epithelia. The vestibular defects were classified as small if the vestibular/dorsal region of the inner ear was smaller than the control pT2K-Hes5-d2EGFP transfected ears (and untransfected ears). Mild phenotype (m) = 5/6 identifiable distinct sensory patches, small vestibular region, cochlea present, and high/low EGFP fluorescence. Severe phenotype (s) = <5 identifiable distinct sensory patches or fused patches, small vestibular region (smaller than the mild phenotype), small or absent cochlea and high/low/no EGFP fluorescence. If it was difficult to accurately identify patches or if patches were fused these marked with an \*.

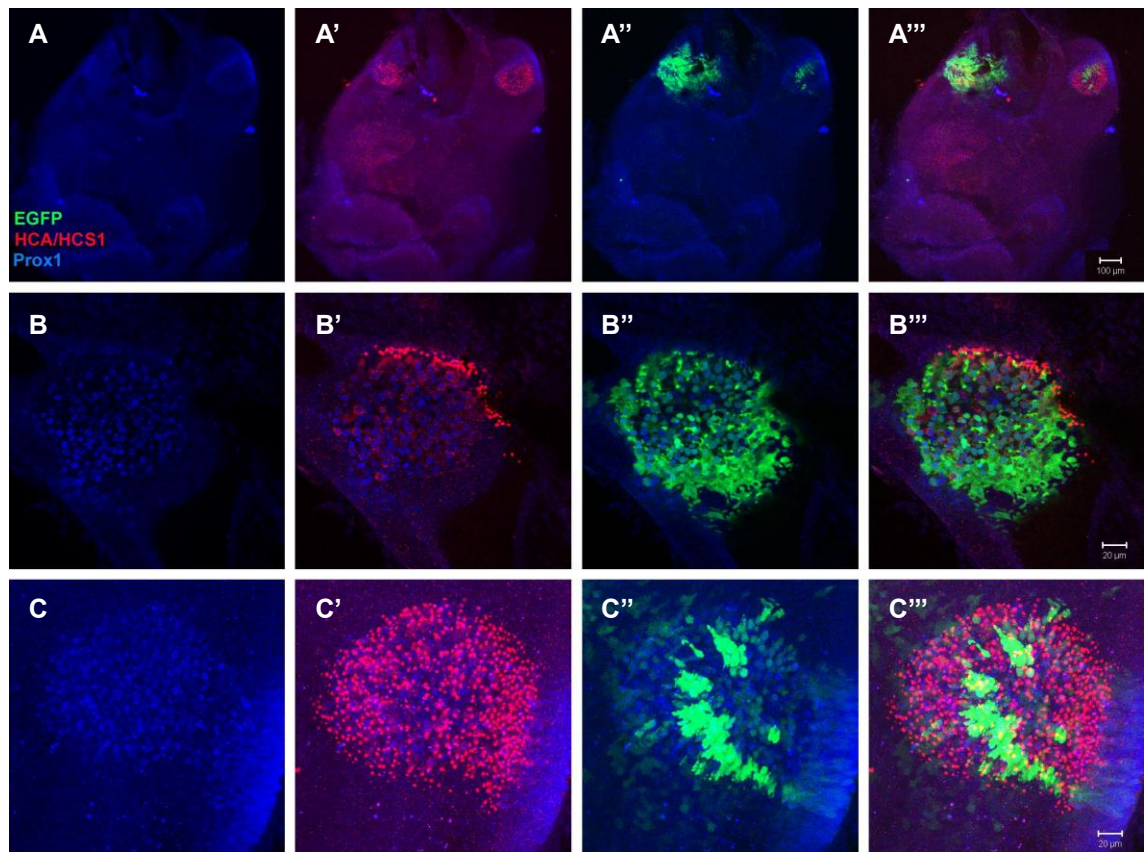
### **5.2.2 Abnormal sensory patch formation in *Hes5-Delta1* transfected ears**

To examine in more details how the construct affected the number, size and morphology of the developing sensory patches, whole-mount transfected inner ears were immunostained with HCA, HCS1, and Prox1 antibodies. The number of patches, within the severely affected ears, varied from 1-5 (see Table 5.1). There were no ectopic sensory patches. There was no indication of expansion of the transfected sensory patches at their borders; on the contrary, the remaining sensory patches appeared smaller than normal (See E11 crista in Figure 5.5 E-E'). The region of Prox1 expression had a regular border and overlapped with that of hair cell immunostaining (Figure 5.6). However in some cases it was very difficult to identify each of the individual sensory patches and so the number of sensory patches within these examples were unknown; these were marked with an asterisk (\*).



**Figure 5.5 PT2K-Hes5-Delta1-IRES-EGFP transfected E8 and E11 ears.** PT2K-Hes5-Delta1-IRES-EGFP was electroporated at E2. Embryos were harvested at E8 and E11 then stained with anti-HCA (red). Control untransfected ears were stained with anti-Serrate1 (green) and/or anti-HCA (**A and E**). PT2K-Hes5-Delta1-IRES-EGFP transfected ears at E8 and E11 with severe abnormalities (**B-D and F**). These ears are smaller and consist of fewer sensory patches (1 and 2 appear to be crista-like structures and 3 is utricle-like), no cochlear duct was present. **E and F** were imaged using the same magnification and show that sensory patches were typically smaller in the transfected ears.





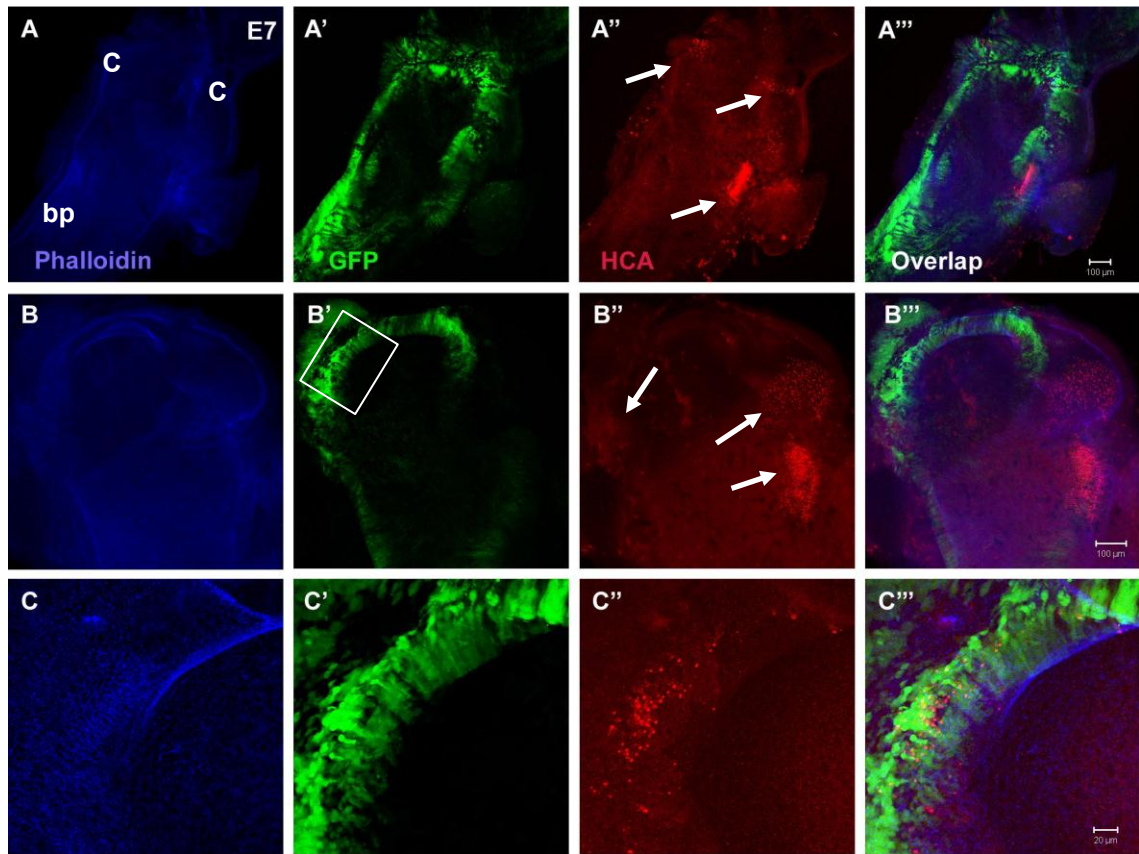
**Figure 5.6 Sensory epithelia transfected with PT2K-Hes5-Delta1-IRES-EGFP.** (A) The gross morphology of E8 the inner ear is abnormal. The basilar papilla is absent and two cristae appear distinct. (B,C) High magnification of the two transfected crista in A. Prox1 (blue) and HCA/HCS1 (red) staining indicates that there is no expansion of the transfected patch. Scale bars = 20µm

It was difficult to identify distinct sensory patches amongst these transfected ears because it appeared as though some patches were fused. In 17 out of 31 cases there was EGFP expression in distinct sensory patches, however, in the remaining 14 there was a continuous band of strong EGFP expression within the dorsal vestibular region. This feature was observed at stages E7, E8 and E14 (figure 5.7, 5.8 and 5.4B). Immunostaining with HCA/HCS1 antibodies showed that ectopic hair cell formation occurred in this ectopic region of EGFP expression. Long continuous bands of hair cells were observed with reduced or complete absence of non-sensory regions that are normally present in the control ears (figure 5.7 and 5.8). The regions with ectopic hair cells were also Prox1 positive, indicating prosensory character. This type of defect suggests that either sensory patches failed to separate at earlier stages or there was a gradual expansion of the sensory patches towards non-sensory regions during their development. It was also noted that within these ectopic sensory regions, there were groups of highly transfected cells that exhibited a reduction in hair cell density (figure 5.8). These specific effects of Hes5-Delta1 on hair cell differentiation are described further in section 5.3.

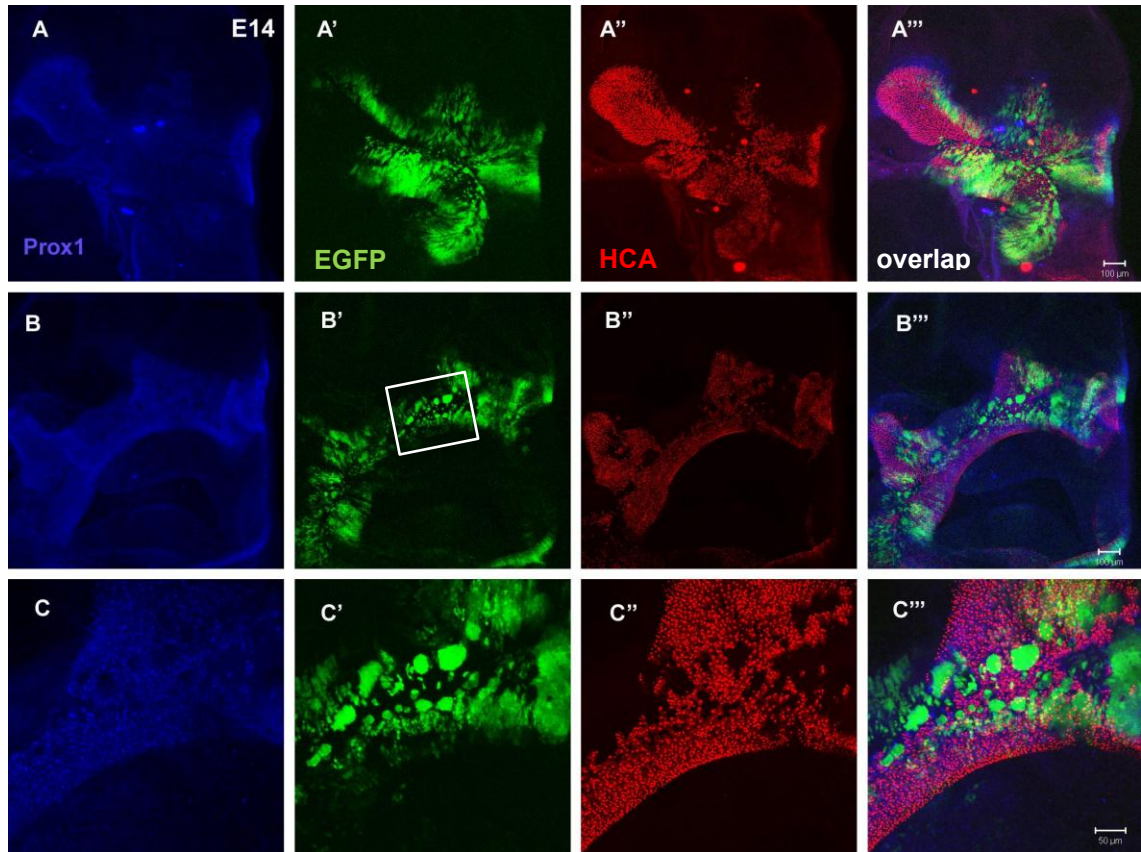
To find out if the defects in inner ear morphology were also present at an early stage of development, I examined the pattern of EGFP expression in two E5 transfected samples. In both samples, there was strong EGFP expression in the crista regions where there would normally be high Notch activity. In addition there was also a widespread and weaker EGFP expression in the vestibular region that did not appear to correspond to any distinctive sensory patch (Figure 5.9 n=2 in one experiment). The HCA staining within this ectopic region of transfection suggests that there could be ectopic hair cell formation (Figure 5.9D). Despite this widespread transfection, the general morphology and size of the ear appeared to be fairly normal at this stage of development. In contrast, the control E5 ears transfected with pT2K-Hes5-d2EGFP showed restricted EGFP expression within prospective sensory regions only. Although this result was obtained in one experiment only and remains preliminary, it suggests that high levels of Notch activity, within non-sensory regions, does not affect inner ear morphology at this stage and thus it is reasonable to suggest that a down regulation of Notch activity is required at a later stage for normal development of the inner ear.

In conclusion, it appears that sustained Notch activity through Delta1 overexpression within sensory patches leads to abnormal sensory patch

development and gross morphology defects of the inner ear especially within the vestibular region of the ear. The most striking consequence of the gain of lateral induction is the appearance of ectopic sensory regions between the endogenous patches, which suggests that lateral induction needs to be down-regulated for normal inner ear morphogenesis and the formation of distinct sensory patches.

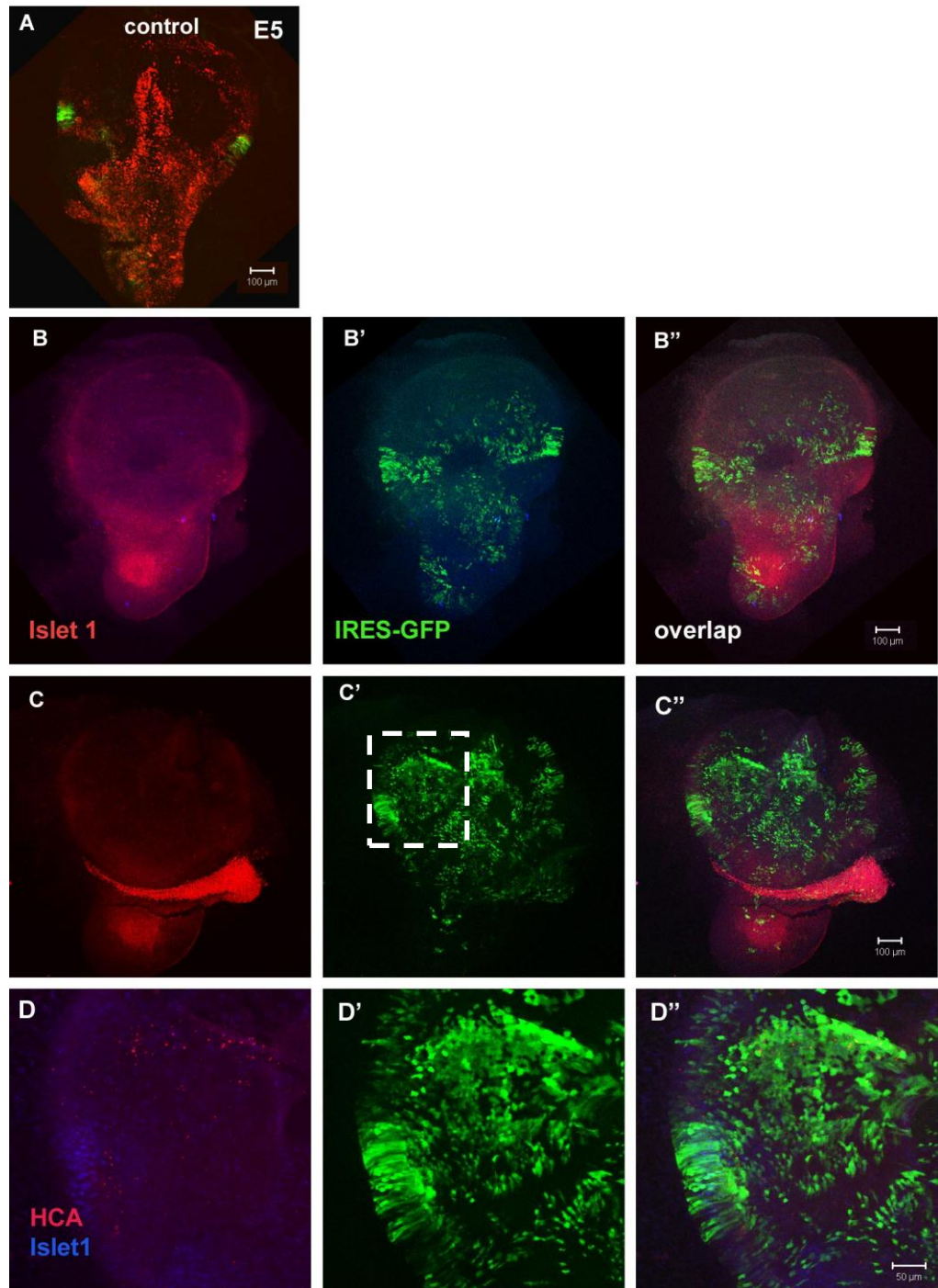


**Figure 5.7 PT2K-Hes5-Delta1-IRES-EGFP transfected E7 ears.** Two transfected ears stained with Phalloidin (blue) and HCA (red) show expanded regions of EGFP across the vestibular domain (**A and B**). The vestibular region is smaller with fewer and smaller sensory patches (white arrows). There are ectopic hair cells formed with the region where there is ectopic activation of the construct in B' (**C''**) (n=6).



**Figure 5.8 PT2K-Hes5-Delta1-IRES-EGFP transfected E14 ears.** Two ears with expanded regions of EGFP across the vestibular domain (**A and B**). The vestibular region is smaller with fewer and smaller sensory patches which appear fused. There are hair cells formed within the region where there is ectopic activation of the construct in **B (C'')**. Prox1 (blue) is expressed in ectopic regions that overlapped with EGFP expression (**B and C**) ( $n = 6$ ).





**Figure 5.9 PT2K-Hes5-Delta1-IRES-EGFP transfected E5 ears.** (A) E5 control co-transfected with pT2K-TomatoNLS and PT2K-Hes5-d2EGFP. (B and C) E5 otocysts transfected with PT2K-Hes5-Delta1-IRES-EGFP, followed by Islet1 and HCA immunostaining. There is strong GFP expression within the regions of the sensory crista and weaker ectopic GFP expression throughout the dorsal region. (D) High magnification of crista in (C) shows ectopic hair cells within non-sensory transfected regions.

## 5.3 The role of Delta1 during hair cell fate decisions

At a later stage of development, lateral inhibition via the ligand Delta1 regulates hair cell versus supporting cell fate decisions. Many loss-of-function studies have shown evidence for lateral inhibition in the inner ear. Mutations that affect the function of the Notch ligand DeltaA in zebrafish result in an excess and premature hair cell production at the expense of supporting cells (Haddon et al., 1998; Riley et al., 1999). In mouse, loss of Delta1 function also leads to excess hair cell differentiation and it is believed that this ligand is the main player during the lateral inhibition of hair cell formation (Brooker et al., 2006; Kiernan et al., 2005a). Surprisingly, gain-of-function experiments in the chicken inner ear failed to demonstrate a role for Delta1 in the inhibition of hair cell formation (Eddison et al., 2000). Here, I found that the normal pattern of hair cell differentiation was clearly disrupted by Hes5-Delta1 transfection.

### 5.3.1 *Transfection with Hes5-Delta1 inhibits hair cell differentiation in trans*

A prediction of the lateral inhibition model is that cells that upregulate Delta1 and that are not themselves exposed to Delta1 should differentiate into hair cells. In contrast, a uniform overexpression of Delta1 should maintain a uniform and high Notch activation and hence lead to a reduction in hair cell formation. To test these predictions, the pT2K-Hes5-Delta1-IRES-GFP construct was electroporated at E2 and allowed to develop to E8-E11, at stages when hair cells have already differentiated in the vestibular sensory patches and in the basilar papilla. Transfected cells were identified by direct GFP fluorescence, and HCA/HCS1 immunostaining was used to detect hair cells. The transfection pattern was mosaic but this proved useful to decipher the effects of Delta1 overexpression in *cis* and in *trans*.

The first observation was that within regions where there were large groups of contacting cells transfected with the construct and expressing high levels of GFP, there was a reduction in hair cell density (Figure 5.10). The hair cell density within the adjacent untransfected regions of the epithelium remained unaffected. This effect was observed in the crista and in the basilar papilla at E8 and E11. This was seen in 23 samples of sensory epithelium in 5 separate experiments. The data in figure 5.10 indicate that within transfection regions of E11 basilar papillae, there was a clear reduction in hair cell density. In the pT2K-Hes5-d2EGFP controls there was no such reduction within the transfected regions (Figure 5.10). It was easier

to visualise the effects on hair cell density in the basilar papilla because the arrangement of hair cells and support cells is more regular than within the vestibular sensory epithelia. A reduction of hair cell formation in a region of high Delta1 expression was predicted as a result of high levels of lateral inhibition. This result confirmed the role of Delta1-mediated lateral inhibition in the regulation of hair cell differentiation.

### **5.3.2 Transfection with Hes5-Delta1 promotes hair cell differentiation in cis**

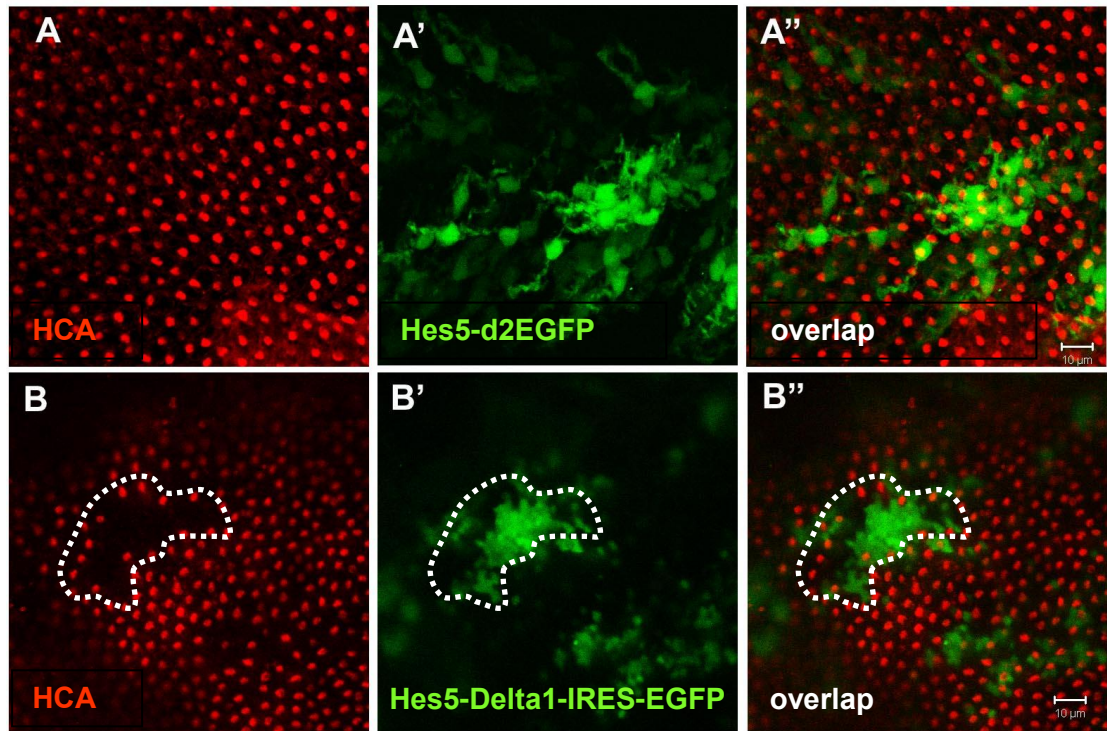
The effects on cell differentiation were very different when transfected cells were isolated from one another. In such cases, a significant number of transfected cells were hair cells. These had usually a low level of GFP fluorescence when compared to supporting/progenitor cells (Figure 5.11 and 5.12). This could be explained by the fact that hair cells normally escape Notch activity and the Hes5 promoter would be inactive. Hence, transfected hair cells are no longer producing GFP and exogenous Delta1 at the time of analysis. By contrast, in transfected supporting cells in which Notch activity is maintained, the fluorescence level remains high.

In order to quantify the effects of Delta1 expression on hair cell and support cell differentiation, the total number of transfected, GFP-positive cells that had differentiated into hair cells and supporting cells were counted within E8 or E11 crista and basilar papilla. Examples of regions within transfected sensory patches are shown in figure 5.11. The results of the cell-counts showed that within 6 different samples, 46% of transfected cells ( $n = 103$ ) within E8 cristae were hair cells, 36% in E11 cristae ( $n = 170$ ) and 48% in E11 basilar papilla ( $n = 764$ ) (as a ratio of hair cells to supporting cells: 1: 1.19, 1: 1.74 and 1:1 respectively). Next, isolated transfected cells in E11 basilar papilla were analysed. A significant proportion of isolated transfected cells, with lower levels of GFP, expressed hair cell markers, HCA and HCS1. It appears there was a clear bias towards the hair cell fate as out of 171 cells, 114 were hair cells (66% hair cells). In E11-E12 bps, which were transfected with a control, pT2K-TomatoNLS, the percentage of transfected cells that were positive for hair cell markers was 30% ( $n = 355$  cells; ratio 1:2.45). This value gives an indication of the normal ratio of hair cells: supporting cells within the basilar papilla at E11-E12 and it lies within the range previously determined by Goodyear and Richardson (1997), in basilar papilla of E12 chicken embryos, 1:1.71 to 1:3.9 depending on the region.

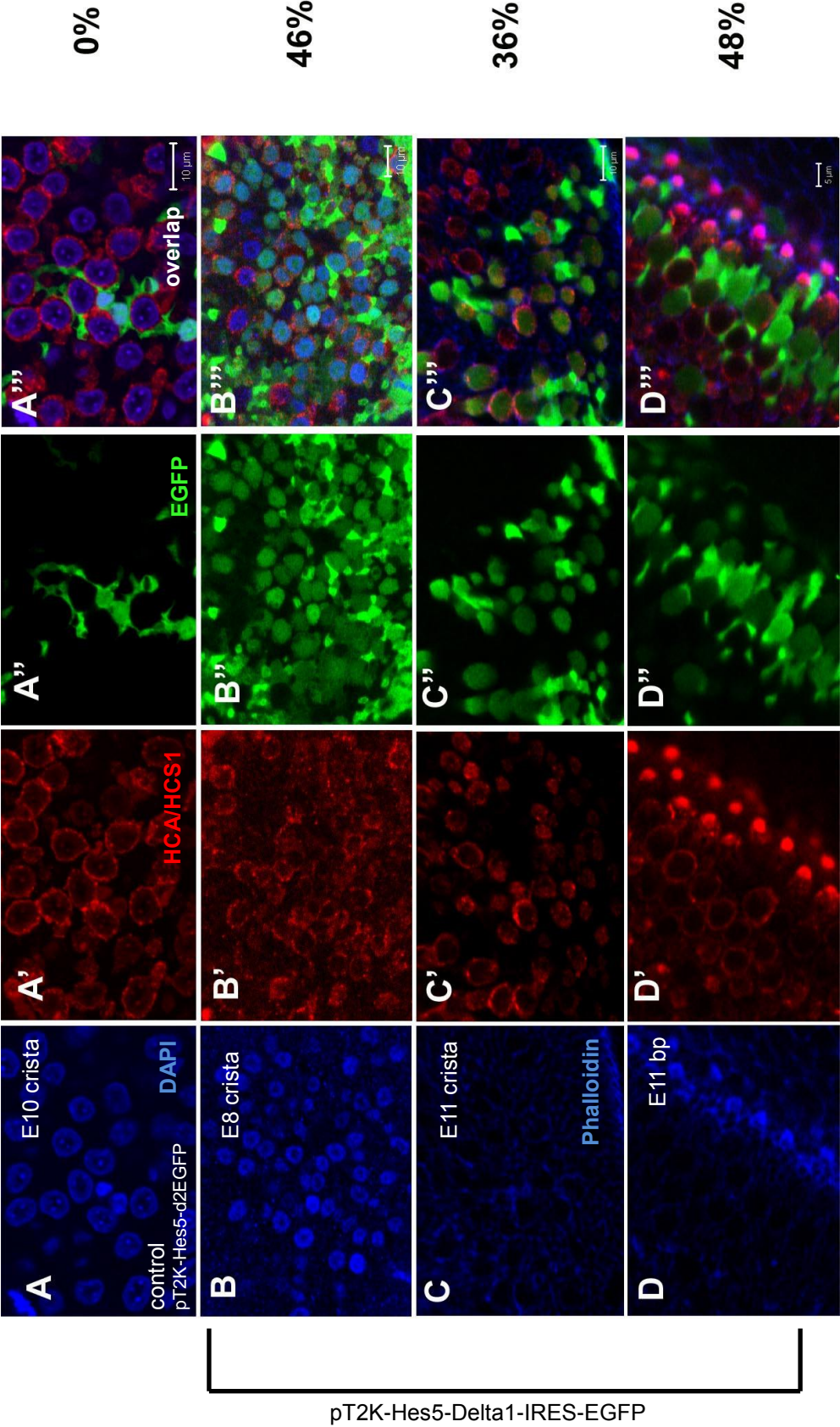


One surprising observation is that, even though the overall hair cell density was greatly reduced, there were still some differentiated hair cells within highly transfected regions. Transfected and untransfected hair cells could also be found adjacent to one or more transfected supporting cells (Figure 5.12B,C). These results suggest that hair cell differentiation could still occur even if strong inhibitory signals are being presented.

Does Delta1 expression control the timing of hair cell formation? One transfected specimen was analysed at E6 - a stage at which hair cells start to differentiate in the apical region of the basilar papilla, but are absent from its basal part (Goodyear and Richardson 1997). In this sample, there was no sign of precocious expression of HCA and HCS1 in the basal region of the basilar papilla where some transfected cells were located. Although these results are very preliminary, they suggest that the upregulation of Delta1 expression is not sufficient to induce premature hair cell formation.

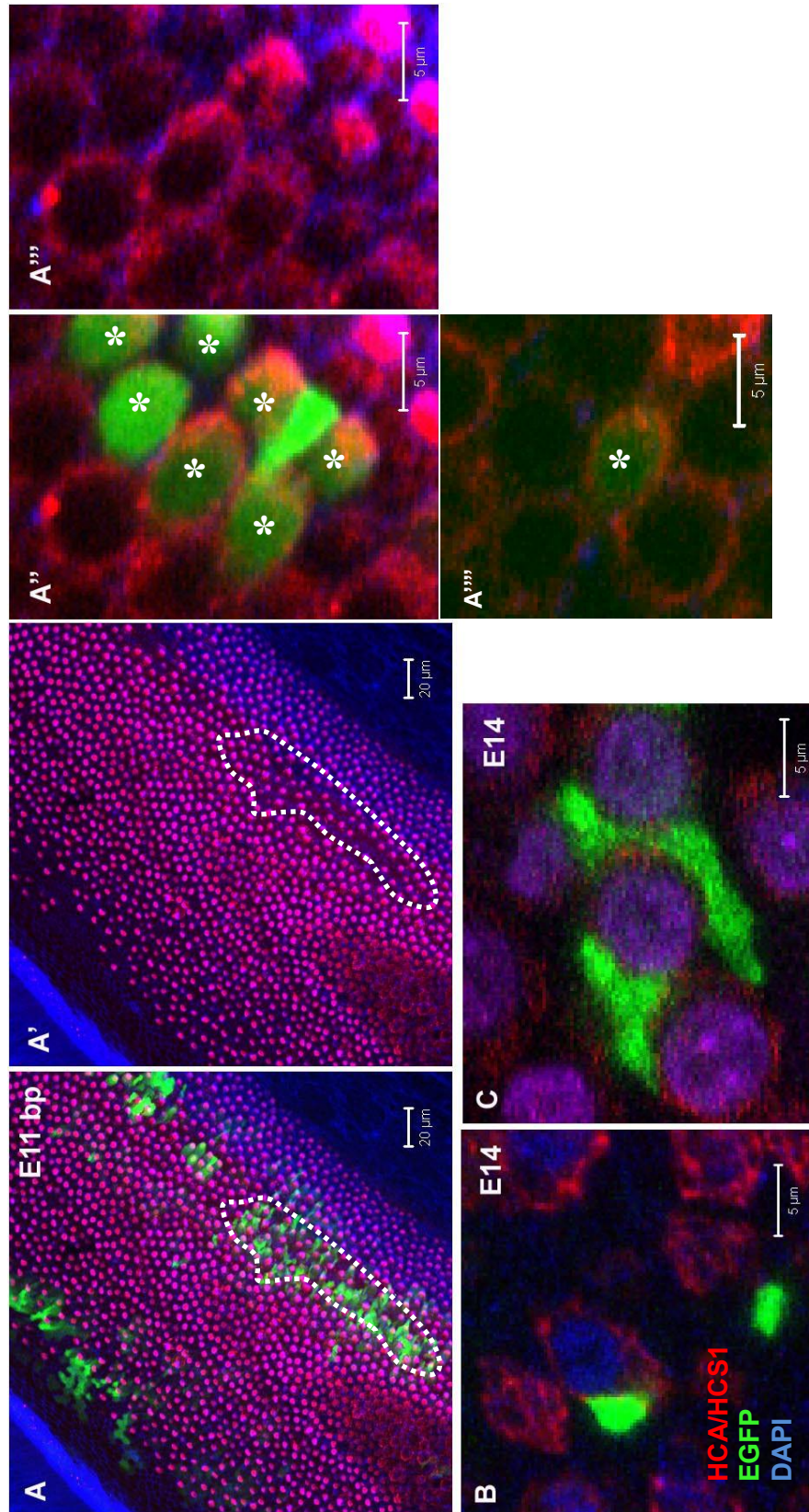


**Figure 5.10 Comparison of the effect of PT2K-Hes5-Delta1-IRES-EGFP transfected E11 basilar papilla. (A-A'')** Regions of bp transfected with PT2K-Hes5d2EGFP (control). There is no reduction of hair cells within the GFP positive regions as shown with the HCA staining. **(B-B'')** shows clearly that within regions of PT2K-Hes5-Delta1-IRES-EGFP transfection there is reduction in hair cell density (white dotted region) (n=23).

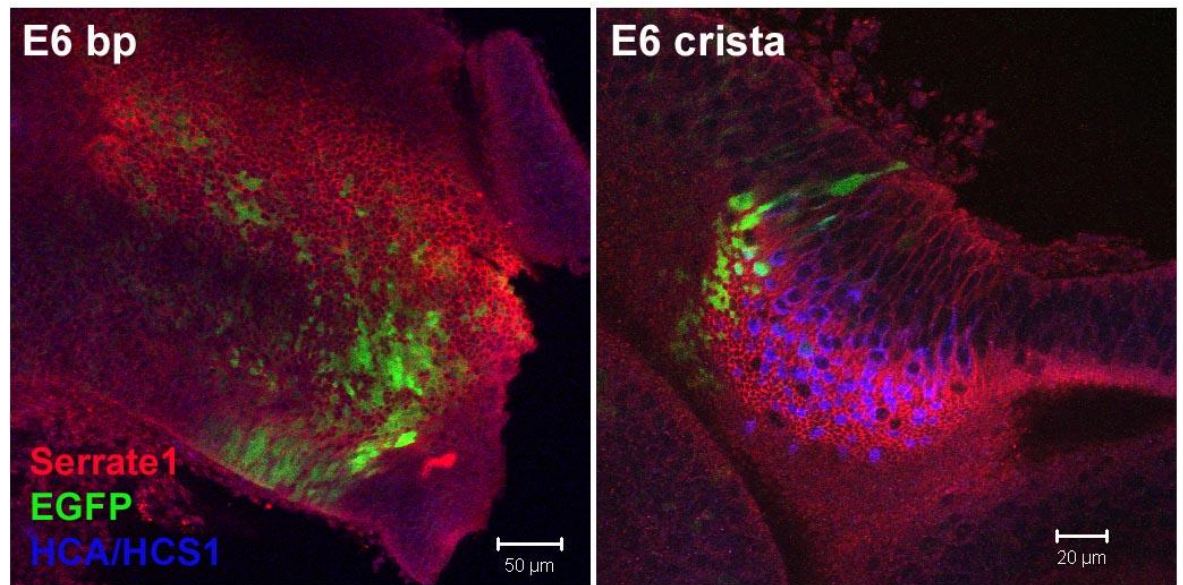


**Figure 5.11 PT2K-Hes5-Delta1-IRES-EGFP is expressed in hair cells. (A-A''') PT2K-Hes5d2EGFP is active in supporting cells/precursor only and not in the hair cells. (B-B''') Shows a region within an E8 crista transfected with PT2K-Hes5-Delta1-IRES-EGFP (C-C''') E11 crista (D-D''') E11 basilar papilla. Hair cells have a weaker level of EGFP expression than the surrounding supporting cells.**





**Figure 5.12 Mosaic transfection of PT2K-Hes5-Delta1-IRES-EGFP results in different cell fate outcomes.** (A-A') E11 bp indicating a reduction in hair cell density within a region of transfection (white dotted region). (A'', A''') Hair cells can form within these regions of transfection where cells are contacting and within isolated cells (A'''). (B and C). Examples of hair cells surrounded by different number of transfection supporting cells showing that hair cell can form even with strong Delta1 inhibitory signals. Scale bars as shown



**Figure 5.13** PT2K-Hes5-Delta1-IRES-EGFP does not induce precocious hair cells in the basilar papilla. There appears to be no hair cell staining in the mid-proximal region of the basilar papilla at E6 (n=1).

# 6

## DISCUSSION

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This thesis has outlined two different approaches to study the functions of the Notch signalling pathway during inner ear development. The first aim was to develop tools to visualise the dynamics of Notch activity in living cells during inner development and the second aim was to test the role of lateral induction using a gain-of-function approach, in which the previously characterised Hes5 promoter was used to drive Delta1. These experiments provide new insights into the role of lateral induction in regulating patch formation and the role that Delta1 plays during lateral inhibition. The new experimental techniques used throughout this project and the main results will be discussed below.

### **6.1 Direct visualisation of the spatio-temporal pattern of Notch activity in the chick inner ear**

#### **6.1.1 Reporters of Notch activity**

Various mechanisms operate to limit the duration of activation of the Notch signalling pathway within cells (Fior and Henrique, 2005; Fior and Henrique, 2009; Fryer et al., 2004; Jarriault et al., 1995). Some mechanisms modulate the availability and quality of receptors and ligands at the cell surface, which in turn affect the strength and duration of signal. After Notch is activated, there are also downstream mechanisms, within the nucleus, that positively or negatively regulate the transcriptional response to Notch. In the absence of Notch, the DNA-binding protein CSL prevents the transcription of Notch target genes. The initiation of transcription relies on the relief of the repression which is brought about by NICD-CSL complex. NICD is then rapidly turned over by the co-factor Mastermind (MAM), this allows for the tight control of the level and timing of Notch signalling. The main target genes for Notch, the HES family of bHLH transcriptional

repressors, are also subjected to tight temporal regulation (Kageyama et al., 2007). HES proteins are not only short-lived but are also able to repress transcription of their own genes (Hirata et al., 2002). Examples of these transcriptional feedback loops have been observed during somitogenesis (Masamizu et al., 2006) and neurogenesis (Kageyama et al., 2008). These regulatory mechanisms could be critical for the temporal and spatial responses to Notch in diverse context.

Notch activation can be studied using antibodies against the cleaved NICD (Tokunaga et al., 2004) and Notch effectors such as *Hes5*. However this only tells you which cells have activated Notch signalling at a given time-point thus limited to retrospective analysis on fixed tissue. Therefore this experimental system cannot be used to detect Notch activation in living tissue or to analyse the fate decision of Notch-active cells in a prospective manner. Therefore reporter assays based on fluorescent or bioluminescent proteins have been developed in order to record Notch signalling in living tissue with high temporal and spatial specificity. Destabilised versions of EGFP (half-life~1-2 hours) and luciferase (half-life~10 minutes) can be useful for reflecting actual transcriptional states of a gene of interest. Although ubiquitinated versions of luciferase have a faster half-life than EGFP, collection of bioluminescence signals requires long exposure time during imaging. This is disadvantageous for high resolution imaging of cell morphology and rapidly moving cells, therefore reporters encoding destabilised fluorescent proteins were selected for this study.

Two main types of fluorescent reporters of Notch activity have been used in previous studies: (1) Reporters with synthetic promoters consisting of multiple CBS (CSL binding sites) (Hansson et al., 2005; Kohyama et al., 2005) and (2) reporters based on the *cis*-regulatory elements of endogenous Notch target genes such as *Hes1* and *Hes5* (Kageyama et al., 2008; Shimojo et al., 2008; Nelson et al., 2006; Ong et al., 2006; Kohyama et al., 2005). These reporters have been used to investigate the role of Notch signalling during cell fate decisions during CNS, retina and kidney development and also they have been used to monitor oscillations in *Hes1* expression during somitogenesis (Masamizu et al., 2006). However this is the first study to use such fluorescent reporters in the embryonic chicken inner ear. The two reporters that were initially tested were the pCBF-d2 Venus YFP and the pHes5-d2EGFP.

### **6.1.2 Characterisation of the reporters by *in ovo* electroporation**

Chicken embryos are easily accessible and offer several advantages for developing a rapid assay to characterise the ability of cis-regulatory elements to promote transcription in specific cell types during development (Uchikawa et al., 2003). The chicken embryo is very easy to manipulate and it is amenable to transfection by *in ovo* electroporation.

At 2 days of incubation the otic placode invaginates to form the otic cup which can be easily filled with DNA solution and transfected. In order to assess the efficiency of transfection, reporters were co-electroporated with a plasmid encoding a red fluorescent protein driven by a constitutively active CMV promoter (pDsRed). The two types of reporters: pCBF-d2Venus YFP and pHes5-d2EGFP were electroporated at E2 and their activation patterns were analysed at E3. At this stage, Notch activity and Hes5 expression occurs in the anterior/neurogenic region of the otocyst where lateral inhibition regulates neuroblast formation (Abello et al., 2007; Adam et al., 1998; Alsina et al., 2004; Daudet et al., 2007). This provided a convenient assay for testing the sensitivity of the reporters to endogenous Notch activity.

The results showed that the activation pattern of pHes5-d2EGFP was restricted to the anterior domain, whereas the CBF reporter had a widespread activation pattern. The pattern of activation of the Hes5 reporter overlapped with endogenous Hes5 gene expression. Previous reports have shown that the arrangement, orientation and spacing of the CBS as well as their distance from the transcriptional start site, are important determinants in target selectivity and activation amplitude (Cave et al., 2005; Ong et al., 2006). Comparison of mouse and chick Hes5.1 promoter region show a similarity in both the number and orientation of the CBS. In contrast, the CBF reporter consists of CBS with the same orientation, which may not be as efficiently bound by the CSL complex *in vivo*, in the inner ear. The CBF reporter may have been more sensitive to lower levels of Notch activity occurring outside of this neurogenic domain, which is known to express Notch1 and other Notch target genes Hes1 (Abello et al., 2007). However it is clear from the activation pattern of the pHes5-d2EGFP reporter that it specifically reflects the ligand-dependent Notch activity in which Hes5 is the main target gene. Therefore the pHes5-d2EGFP was used for the rest of the investigation. Overexpression of Delta1 and NICD leads to ectopic reporter activity throughout the otic cup. These results provided further evidence that the reporter is sensitive to Notch activity.



Signalling pathways other than Notch may contribute to the activation of the Hes5 reporter. One way to exclude this possibility would have been to test a construct with a mutated form of the Hes5 promoter. In the presence of Notch activation, the wildtype Hes5 reporter activity should be stronger than the one with a mutated promoter. Nevertheless, the specificity of the response of the Hes5 reporter was later confirmed using the  $\gamma$ -secretase inhibitor, DAPT.

### **6.1.3 Long term expression vectors: RCAN and Tol2-mediated gene transfer**

One limitation of using standard plasmid electroporation is that foreign DNA gets diluted and degraded as development proceeds and transfected cells divide. Expression of exogenous genes typically fades after 4-5 days post-electroporation when the plasmid is not integrated within the cells genome. In order to be able to use the reporter to assess Notch activity at later stages of development, when hair cell and supporting cell differentiation is occurring, I cloned the reporter into two different vectors: RCAN retroviral vector and the Tol2 transposon. Both constructs elicited stable integration of the Hes5 reporter, which was specifically activated within the sensory epithelia of the inner ear.

At the time when the study began, the preferred method for achieving long term expression of exogenous genes in chicken cells was using the RCAS retrovirus, which was developed by Hughes et al. (1987). Previous studies have used RCAS infection to transfer genes into the inner ear (Eddison et al., 2000; Kiernan and Fekete, 1997) but the first study to use in ovo electroporation to transfect the proviral RCAS DNA was by Bird et al. (2010). This study showed that electroporation of the otic cup can lead to sustained gene expression in the chick inner ear up to E19. The cells that are transfected with retroviral DNA produce infectious particles, which could in turn infect more cells, resulting in a greater number of cells inheriting the transgene.

In this study, I found that the RCAN derivative of RCAS, which allows the gene of interest to be driven by a specific promoter, could also be used to successfully achieve stable integration of a transgene within the chick inner ear via in ovo electroporation. An advantage of using such a vector is that infected cells become refractory to further infection, so therefore only one copy of the transgene would be integrated per cell. This could facilitate

comparison of fluorescence levels from cell to cell. However there are some important limitations to using RCAS vectors. They cannot carry transgenes which are larger than 2.5kb and due to cells becoming refractory to secondary infection it is not possible to co-express different transgenes unless retroviruses harbouring different envelope proteins are used. Another important limitation is that the time of infection for any given cell is not known. One way to overcome this problem could have been to use a replication defective vector, however at the time, the Tol2 transposon system appeared as a better alternative. These vectors originate from the Tol2-transposon of the Medaka fish (Koga et al., 1996) and have been used for transgenesis in different animals. Recently Tol2-transposon vectors have been shown to successfully achieve gene transfer in avian cells (Sato et al., 2007; Watanabe et al., 2007).

The Tol2 vectors had several advantages over retrovirus for my experiments. Firstly, it is possible to co-electroporate several Tol2 plasmids at the same time. In this study, a control plasmid, pT2K-TomatoNLS, was co-electroporated to mark the transfected cells along with the pT2K-Hes5-d2EGFP reporter. This was particularly important for identifying the cells that had been transfected, but were no longer Notch active and hence did not exhibit any GFP fluorescence. In the live imaging experiments it became apparent that many of the GFP-negative cells with a flask-shape had an apically localised nucleus, characteristic of hair cells. Secondly, Tol2 vectors have a capacity to carry a much larger transgenes (up to 11kb) than RCAS vectors. This proved absolutely essential for the second part of my work, in which the Delta1-IRES-GFP sequence needed to be expressed downstream of the Hes5 promoter region (see section 6.2).

#### ***6.1.4 Is the Hes5 reporter activated during Serrate1-mediated lateral induction and Delta1-mediated lateral inhibition?***

There are several Notch ligands expressed in the early embryonic inner ear, which could lead to the activation of the Hes5 reporter. Serrate1 (also named Jagged1 in mammals), is expressed initially in the posterior-medial region of the otic placode at E2 and then resolves into two poles, anterior and posterior, which are connected by a medial domain of weaker Serrate1 expression. It then becomes restricted to sensory patches where it is expressed within progenitor and supporting cells, but not hair cells (Abello et al., 2007; Adam et al., 1998; Cole et al., 2000; Myat et al., 1996). Serrate1 is known to be important for early formation of sensory domains (Brooker et al., 2006; Kiernan et al., 2006; Daudet et al., 2007). The evidence to date

suggests that an early phase of Notch activity mediated via Serrate1 operates through lateral induction whereby a cell expressing Serrate1 will activate Notch and induce Serrate1 expression in the adjacent cells, creating a positive-feedback loop (Daudet and Lewis, 2005; Eddison et al., 2000).

Delta1 is expressed in the neurogenic domain of the otic vesicle and in nascent hair cells and mediates lateral inhibition (Adam et al., 1998; Morrison et al., 1999). In addition to Delta1, Jagged2 is later expressed in nascent hair cells (Lewis et al., 1998; Lanford et al., 1999). The expression pattern of Hes5 has been shown previously to be complementary to Delta1 expression and depends on Notch activation (Abello et al., 2007; Daudet et al., 2007; Neves et al., 2011).

Despite these differences in function and pattern of expression, it is assumed that all these Notch ligands are able to elicit activation of Notch1, which is the only Notch receptor expressed in the chicken inner ear (Adam et al., 1998). Therefore the activity of the Hes5 reporter could be a representation of the combined input of these two ligands on Notch1 activity. In support of this idea, I found that the reporter is active in the posterior region of the otocyst at stages when Serrate1, but not Delta1, is expressed. In addition the reporter is sensitive to artificial Notch1 ICD that is thought to be mediating lateral induction and inhibition in the chicken inner ear (Daudet et al 2005, see lateral induction section).

However, there might be qualitative differences in the abilities of Delta1 and Serrate1 to activate Notch1 and the Hes5 reporter. Notch receptors could potentially be more or less sensitive to a specific ligand via post-translational modifications. Fringe is one possible modulator that could both positively and negatively modulate the ability of Notch ligands to activate Notch signalling (Moloney et al., 2000; Panin et al., 1997). It has been shown to inhibit Serrate-induced and potentiate Delta-induced Notch signalling at the dorsal/ventral boundary of the wing imaginal disk in *Drosophila* wing (Fleming et al., 1997; Panin et al., 1997). It also has positioned Notch activation in early oogenesis and in developing legs and eye (Grammont and Irvine, 2001; Irvine, 1999).

A recent study using chick otic vesicles has shown, by quantitative RT-PCR, that the target genes for Serrate1-mediated Notch activation are *Hes1*, *Hey1* and *Hey2*, but not the *Hes5* gene (Neves et al., 2011). This suggests that Hes5 expression is associated with Delta1-mediated lateral inhibition, but not with Serrate1-mediated lateral induction. The absence of Hes5 induction

by Serrate1 could mean that the Hes5 reporter is not able to detect Notch activity during both lateral induction and inhibition and perhaps solely marks Delta1-mediated Notch activity. The results from this study provide some further indication that the Hes5 reporter might indeed be more strongly activated by Delta1 than by Serrate1. In fact, the reporter was more strongly active in the cristae where hair cell differentiation occurs first and weaker in the basilar papilla and other vestibular patches where hair cell differentiation occurs later on. However this might reflect quantitative rather than qualitative differences: at the time of hair cell formation, the combined input of Delta1 and Serrate1 could explain the higher activity of the Hes5 reporter.

Although there is sufficient evidence to confirm that the Hes5 reporter can detect Delta1-mediated Notch activity, it still remains unclear as to whether the reporter can detect with the same efficiency Serrate1-mediated Notch activity. One possible experiment to address this question could be to co-transfect the Hes5 reporter and an RCAS-Serrate1 construct into the otic cup. If there is ectopic activation of the reporter, as seen with the RCAS-Delta1 construct, then it could be assumed that this reporter can detect Notch activity irrespective of the ligand involved.

#### **6.1.5 Dynamics of Notch activity at the time of hair cell formation**

Mathematical modelling has shown how cell fate decisions and tissue patterning occurs by lateral inhibition. However there is limited *in vivo* data of how this occurs. This type of modelling work may not account for certain cellular processes that may also be occurring such as cell proliferation, cell death and rearrangements which ensure that the correct number of cells are produced at the correct time and place. One aim of the study was to determine whether a genetic reporter could be used to monitor the dynamics of Notch activity during hair cell differentiation.

My data show both the RCAN and the Tol2 transposon vectors can be used to achieve stable integration of the Hes5-d2EGFP reporter into the otic cup via electroporation. Reporter activity persisted until at least E10 and E16, respectively. I found that the Hes5-d2EGFP reporter exhibited a discrete spatial pattern of activation that is consistent with the endogenous pattern of *Hes5* genes expression. It is specifically active in the progenitor and supporting cells and not in differentiated hair cells.

The lack of activation of the reporter in hair cells indicates that the reporter is switched off when cells are no longer experiencing Notch activation. A similar result was found in the retinal ganglion cells, where a loss of Hes gene expression and reporter activity indicates that Notch activity is inhibited at some point prior to this final division (Nelson et al., 2006). The loss of Notch signalling in differentiating neurons and hair cell is consistent with the known role of the Notch signalling pathway in regulating cell fate decisions by maintaining the undifferentiated state of the progenitor cells via lateral inhibition.

Live-imaging of the reporter at high magnification showed differences of fluorescence levels between progenitor cells at stages where hair cells are forming, which may reflect differences of Notch activity between individual progenitor cells. Amongst the transfected progenitor cells, some were decreasing in fluorescence whilst others were increasing and these dynamic changes over time could reflect endogenous variations of Notch activity.

However there are several complications that would affect the accurate interpretation of these variations in fluorescence signal. Firstly, transfection was often widespread throughout the sensory patches, making it difficult to distinguish EGFP fluorescence amongst adjacent cells. Secondly, variations of the cells' morphology and volume meant that the apparent variations of fluorescence intensity were not always a true representation of the endogenous changes of EGFP expression levels. For example, dividing cells would round up at the apical surface just before mitosis and appear to have higher fluorescence levels in their cytoplasm. Thirdly, the EGFP protein has been shown to be too stable to mimic the endogenous decrease of Hes5 mRNA as shown by the quantitative RT-PCR data. There is delay of ~5 hours from the time when the Hes5 message decreases by 50% to the time when the fluorescence decreases by 50%. These results indicate that this reporter is not dynamic enough to report the transcriptional responses of Notch activity in real-time. A reporter with a much shorter half-life than d2EGFP would be required to do so (Li et al., 1998).

There are also limitations with the live-imaging technique which cannot be overcome, for example photo-bleaching. Controls in this study have shown that bleaching can be kept to a minimum by optimisation of laser intensities. The other major problem with live imaging is movements of cells in the Z-plane which can affect the intensity of fluorescence signals and complicates tracking of individual cells.

Stable integration of the reporter meant that the transfected inner ear could be dissected and cultured ex-vivo and thus enabled the analysis of the reporter's dynamic response to a decrease in Notch activity induced by treatment with DAPT, a  $\gamma$ -secretase inhibitor. DAPT was initially developed to inhibit amyloid precursor protein processing for investigating possible therapeutic strategies for Alzheimer's disease (Dovey et al., 2001). As  $\gamma$ -secretase activity is required for proteolysis of Notch receptor and the release of NICD, it has been used as a pharmacological tool for inhibiting Notch activity. The phenotypes generated with DAPT are indistinguishable from the phenotypes generated from Notch loss of function phenotypes in vivo in the zebrafish (Geling et al., 2002). DAPT treatment leads to excess hair cell production (Takebayashi et al., 2007) and a reduction in prosensory cell formation in the anterior otocyst (Daudet et al., 2007). It was previously reported that DAPT prevents expression from Notch activity-dependent reporter constructs (Nelson et al., 2006, Ong et al., 2006; Hansson et al., 2006) and was found to be a useful reagent for effectively inhibiting Notch activity without the need for dominant-negative constructs or antisense oligonucleotides approaches. Here I demonstrated that the pHes5-d2EGFP reporter is sensitive to a reduction of Notch activity induced by DAPT so therefore it can be used to monitor ligand-dependent Notch activity.

Although there are some difficulties with analysing the fluorescence levels within single-cells, the Hes5 reporter can be a useful tool. The time-lapse experiments with DAPT have shown it is possible to monitor a unidirectional change in Notch activity. The time-course of reduction in fluorescence in DAPT-treated samples also suggest that the transcriptional response to Notch in progenitor cells is switched off in less than 3.5 hours from when the trans-activation of Notch receptors ceases. However this time is overestimated as the time it takes for DAPT to inhibit  $\gamma$ -secretase and Notch cleavage and the time for d2EGFP mRNA to be degraded, have not been factored in.

#### **6.1.6 Cellular dynamics at the time of hair cell formation**

There have been no reports on the cellular dynamics during hair cell formation. Data from this study shows that at the time of hair cell formation, the sensory epithelium undergoes several dynamic processes during which the Notch-active cells can be monitored and analysed with live-imaging. Processes included: interkinetic nuclear migration and cell addition during extensive proliferation, cell death and cell morphology changes during

division. Within the sensory epithelia, the behaviour of different cell types can be observed. Interkinetic nuclear migrations were clearly seen amongst proliferating Notch-active progenitors and have been noted to be mainly symmetrical division as opposed to asymmetric division; however this needs to be confirmed by quantification. Evidence of vertical nuclear migration occurring during chick inner ear development was first given by Katayama and Corwin (1993). The two identical daughter cells that resulted from mitosis, continued to experience Notch signalling. The differentiated hair cells, with no GFP, can be easily distinguished and thus useful for testing different hypotheses of how different signalling pathways effect differentiation as well as how Notch signalling affects proliferation and differentiation. The cellular rearrangements could provide information about how the final ordered cellular mosaic pattern is established and the potential role of Notch signalling in regulating these processes. Overall this reporter provides insights to the many cellular processes that are occurring within inner ear sensory epithelia that have not yet been recorded with such high resolution.

#### **6.1.7 *Future perspectives***

I have shown that a Hes5 reporter can be stably integrated in the developing chicken inner ear using RCAN or Tol2-mediated gene transfer. This provides an opportunity to investigate how changes in Notch activity are coordinated with other dynamic cellular processes such as cell proliferation and reorganisation during inner ear development. The reporter can also be a useful tool for testing efficiency of agents that activate or block Notch signalling in live cells, as shown here using DAPT. In order to overcome the particular problems of using this cytoplasmic version of the reporter, a nuclear localised and destabilised EGFP reporter could be generated. As the fluorescence would be contained within the nucleus of the cells, it would make individual cells more distinct and easy to track and thus facilitate quantification of fluorescence. A recent study has used a novel reporter based on the chick Hes5.1 promoter to monitor Notch activity during neurogenesis in the chick neural tube. Instability was achieved using a destabilised nuclear Venus fluorescent protein and the 3' UTR of Hes5.1 (Vilas-Boas et al., 2011). It would be interesting to test the activity and dynamic properties of this reporter construct in the chicken inner ear. The use of Tol2 transposon could also enable the co-expression of multiple reporters within the same cell. For example, an Atoh1-reporter would be useful to study how changes in Notch activity relate to commitment to a hair cell fate during lateral inhibition.

Finally, the characterisation of the Hes5 promoter region showed that it could be used to drive the expression of any transgene in a Notch-regulated manner. This approach proved particularly interesting to study the function of lateral induction and Delta1 during inner ear development.

## **6.2 The role of lateral induction during inner ear development**

### ***6.2.1 The role of Notch activity and Sox2 in the early formation of prosensory domains***

Most studies agree that Notch signalling plays a critical role in prosensory formation. The Notch ligand Jagged1 and Lfng, which modulates the binding of Notch ligands to their receptors (Bruckner et al., 2000; Moloney et al., 2000), are expressed in patterns that are consistent with a role in prosensory specification. These genes are initially expressed in a diffuse pattern within the otic cup and eventually become restricted to the prosensory regions (Adam et al., 1998; Cole et al., 2000; Morsli et al., 1998; Wu and Oh, 1996). Jagged1 mouse mutants have a decrease in the size of all sensory patches (Brooker et al., 2006; Kiernan et al., 2001; Kiernan et al., 2006; Tsai et al., 2001). Furthermore it has been shown that overexpression of the activated form of the chicken Notch1 receptor within non-sensory regions of the otocyst has resulted in the formation of ectopic sensory patches (Daudet and Lewis, 2005). Similar findings have been obtained in transgenic mice more recently (Hartman et al., 2010; Pan et al., 2010).

A possible mechanism for sensory organ specification is that Jagged1 is responsible for maintaining Sox2 expression within restricted regions of an extended neurosensory-competent domain of the otic epithelium (Neves et al., 2011). Neves et al. (2011) showed that the Sox2 expression domain is broader than Jagged1 before prosensory domain specification, but then as prosensory patches develop, Sox2 is lost from Jagged1-negative regions. Also when they overexpressed chicken Sox2, it resulted in ectopic sensory and neurogenic patches and transfected domains were expressing differentiated hair cells. The results of their study suggested that Sox2 expression provides the competence to develop into sensory cells and that Notch signalling regulates prosensory specification by its effects on Sox2 expression (Neves et al., 2011).



There has been evidence that Notch positively regulates expression of Jagged1 via lateral induction in early prosensory patches of the inner ear which strengthens and maintains Notch activation and prosensory state. It has been shown that Jagged1 is uniformly expressed within sensory progenitors (Morrison et al., 1999). Jagged1 mutant mice exhibit impaired sensory patch formation rather than the excess hair cell production characteristic of a loss of lateral inhibition. It has also been shown that there is a reduction of Serrate1 in the chick otic epithelium when Notch is inhibited (Daudet et al., 2007). More recently it has been further confirmed that there is a downregulation of the prosensory markers, Sox2, BMP4, Lfng and Hey1 in the inner ear of Jagged1-cko mouse (Pan et al., 2010). Together the data suggests that Jagged1 is positively regulated by Notch and that lateral induction mediated by Jagged1, is important for the early development of sensory patches. But does it promote the expansion of prosensory patches or is it required for the formation of boundaries between sensory and non-sensory regions?

### ***6.2.2 What is the role of lateral induction during prosensory patch development?***

Until recently there has been a lack of gain-of-function studies testing the role of Notch dependent lateral induction. Recent studies have confirmed that Notch activity is sufficient for prosensory specification and induction of ectopic sensory patches (Hartman et al., 2010; Pan et al., 2010). However, these experiments have not established the specific function of lateral induction. In order to investigate the role of lateral induction during chick inner ear development I used a construct (pT2K-Hes5-Delta1-IRES-EGFP) that would drive Delta1 only in Notch active cells and thus created an artificial gain-of-lateral induction. If the role of lateral induction is to promote the expansion/growth of sensory patches then the expected results would be that transfected sensory patches would become larger. My results showed that chick inner ears transfected with the pT2K-Hes5-Delta1-IRES-EGFP construct developed abnormally but the extent of the transfection and phenotype varied greatly.

There were two main phenotypes observed in the pT2K-Hes5-Delta1-IRES-EGFP transfected ears. The first (in 16 out of 31 samples) was characterised by low GFP expression within the sensory epithelia. These ears were considerably smaller than normal ears, had missing basilar papilla and/or a reduced number of vestibular patches. The patches that were present were very small, clearly separated and contained some hair cells. One potential

cause of this phenotype could be extensive cell death as the GFP levels were low within these ears. This aspect could be looked at in the future using apoptotic markers. Another possible explanation for the severity of this abnormal development could be due to the amount of DNA transfected per electroporated ear. This low GFP may be accounted for by either a lack of transposase being transfected, cell death, or the cessation of transcription from the *Hes5* promoter.

The second, more striking, phenotype (in 14 out of 31 samples) consisted of widespread GFP expression throughout the different sensory epithelia. The vestibular regions of these ears were still smaller than controls, but consisted of more sensory patches than observed in the small ears. The basilar papilla looked fairly normal. In most cases, there were ectopic sensory patches within non-sensory regions that appeared continuous with the endogenous patches. This made identifying distinct patches difficult. This phenotype might suggest that lateral induction plays a role in boundary formation during inner ear sensory patch development. Notch signalling regulates boundary formation in *Drosophila* wing formation (de Celis et al., 1996), so therefore one possibility is that it has the same role in the specification of the boundaries of prosensory domains in the inner ear.

Hence, the two consequences of the gain of lateral induction were 1) a reduction in the overall size of transfected patches and 2) the formation of ectopic hair cells in between endogenous sensory patches. These results suggest that lateral induction does not have a direct role in the control of patch size, but rather that it may play a role in the formation of boundaries between sensory and non-sensory domains.

However, it still remains unclear as to whether non-sensory regions are being converted to sensory regions or whether a common prosensory domain has failed to separate to form distinct patches. According to the model suggested by Neves et al. (2011), Notch signalling is required to maintain Sox2 expression within prosensory regions. The data in this study fits the model as the persistent Notch activity leads to ectopic sensory domains. Maintaining high levels of Notch activity, could be preventing the down regulation of Sox2 expression. In order to provide further evidence for this hypothesis, the Sox2 expression pattern within the transfected regions needs to be checked by immunohistochemistry. However, sensory patch marker, *Prox1*, is observed which confirms that this ectopic region of transfection is in fact sensory. One possible hypothesis is that there is an

initial high level of Notch activity within a diffuse region of the otocyst, which is then downregulated in order to restrict Notch activity within the prosensory patches. In order to form any strong conclusions, further investigation is required into this mechanism and the identification of factors that may contribute to the down regulation of the Notch signalling pathway. One possible way to demonstrate whether there is a downregulation of Notch activity might be to transfect the otocyst with an RCAN-Hes5-EGFP construct, in which the EGFP is not destabilised and check various stages of early inner ear development. If this experiment shows that there is ectopic EGFP expression i.e in non-sensory regions, it would support the hypothesis that the down-regulation of Notch signalling is required for the formation of discrete sensory patch boundaries.

### ***6.2.3 Is prosensory determination by lateral induction a Jagged1 – specific mechanism?***

A question that still remains unanswered is whether any Notch ligand, Delta1 or Serrate1, is equally efficient in mediating lateral induction. A recent study has found that overexpression of Delta1 does not induce Serrate1 expression, suggesting that lateral induction is a Jagged1-dependent mechanism (Neves et al., 2011). However, my results show that Delta1 is just as capable as Serrate1 at inducing Serrate1 and ectopic sensory patches. Furthermore, studies have shown that active Notch is sufficient to mimic the effects of Jagged1 in the induction of ectopic prosensory (Hartman et al., 2010; Pan et al., 2010; Daudet and Lewis, 2005) thus suggesting that the prosensory function of Notch is, to some extent, ligand-independent. The fact that Delta1 and Jagged1 play different roles during inner ear development (Brooker et al., 2006; Daudet et al., 2007; Eddison et al., 2000) could be primarily due to the way they interact with the Notch receptor and to their different patterns of expression.

### ***6.2.4 What factors cooperate with Notch signalling to regulate patch boundaries?***

There are several other factors that could interact either directly or indirectly with the Notch signalling pathway in its prosensory function. Lmx1a is a member of the LIM homeodomain (LIM-hd) transcription factors which are critical to cell fate decisions and patterning of organs (Hobert and Westphal, 2000). Lmx1a is expressed initially throughout the otic placode but is then restricted to specific regions of the otocyst (Failli et al., 2002). The function of Lmx1a in the inner ear has been implicated in the *dreher* mutants (Koo et al., 2009). It does not induce neural, sensory and non-

sensory domains but is required for their proper segregation. The lack of *Lmx1a* results in a similar phenotype seen in the severe *Hes5-Delta1* transfected ears i.e abnormal boundaries between vestibular domains. Other factors that are essential for the formation of the vestibular apparatus is *Bmp4* (Chang et al., 2008; Gerlach et al., 2000), FGFs (Chang et al., 2004) and members of the homeobox containing gene families, *Dlx* and *Hmx* (Wang et al., 2004).

Although it is known that Notch maintains *Jagged1* within sensory patches, it still remains unknown as to what factors are involved in the regulation of the initiation of *Jagged1* expression (Daudet et al., 2007). A prime candidate for the regulation of *Jagged1* expression in the prosensory domains is Wnt signalling. Firstly, *Jagged1* is a conserved target of canonical Wnt signalling (Kato, 2006) and secondly, it has been shown that Wnt signalling induces *Serrate1* expression in the otic early epithelium (Jayasena et al., 2008). Similarly to *Hes5-Delta1* transfected ears, overexpression of Wnt signalling results in ectopic and fused sensory patches (Sienknecht and Fekete 2008; Stevens et al., 2003). Therefore Wnt could act upstream of Notch signalling to regulate sensory organ specification in the otic epithelium.

### **6.3 The role of Delta1 in the lateral inhibition of hair cell formation**

Hair cells and support cells are derived from a common pool of precursor cells. The distribution of these two cell types is mostly the same across inner ear epithelia: every cell that contacts a hair cell is a supporting cell and any cell that escapes all contact with hair cells is itself a hair cell. There has been substantial evidence that Notch-mediated lateral inhibition is essential for regulating cell fate decisions within the inner ear sensory patches. This has been the main focus of most studies on Notch signalling in the inner ear. Nascent hair cells express three DSL ligands: *Delta1*, *Delta3* and *Jagged2* (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999; Hartman et al., 2007). The absence of *Delta1* leads to an excess production of neurons and hair cells (Brooker et al., 2006; Kiernan et al., 2005a), which is more dramatic than that seen in either *Jagged2* (Lanford et al., 1999) or *Delta3* (Hartman et al., 2007) mutant mice. Therefore *Delta1* is thought to play the most significant role during the lateral inhibition of hair cell formation. However, there are still some questions remaining regarding the actual function of *Delta1*. It has not been shown directly whether cell-to-cell competition for *Delta1* expression dictates hair cell and supporting cell fate decisions. Furthermore, previous experiments using RCAS retrovirus to

overexpress Delta1 in the chick inner ear have failed to confirm the role of Delta1 in regulating hair cell production (Eddison et al., 2000). Here, the gain-of-function experiments with the pHes5-Delta1-IRES-EGFP construct provided new insights into the mode of action of Delta1 during hair cell fate decisions.

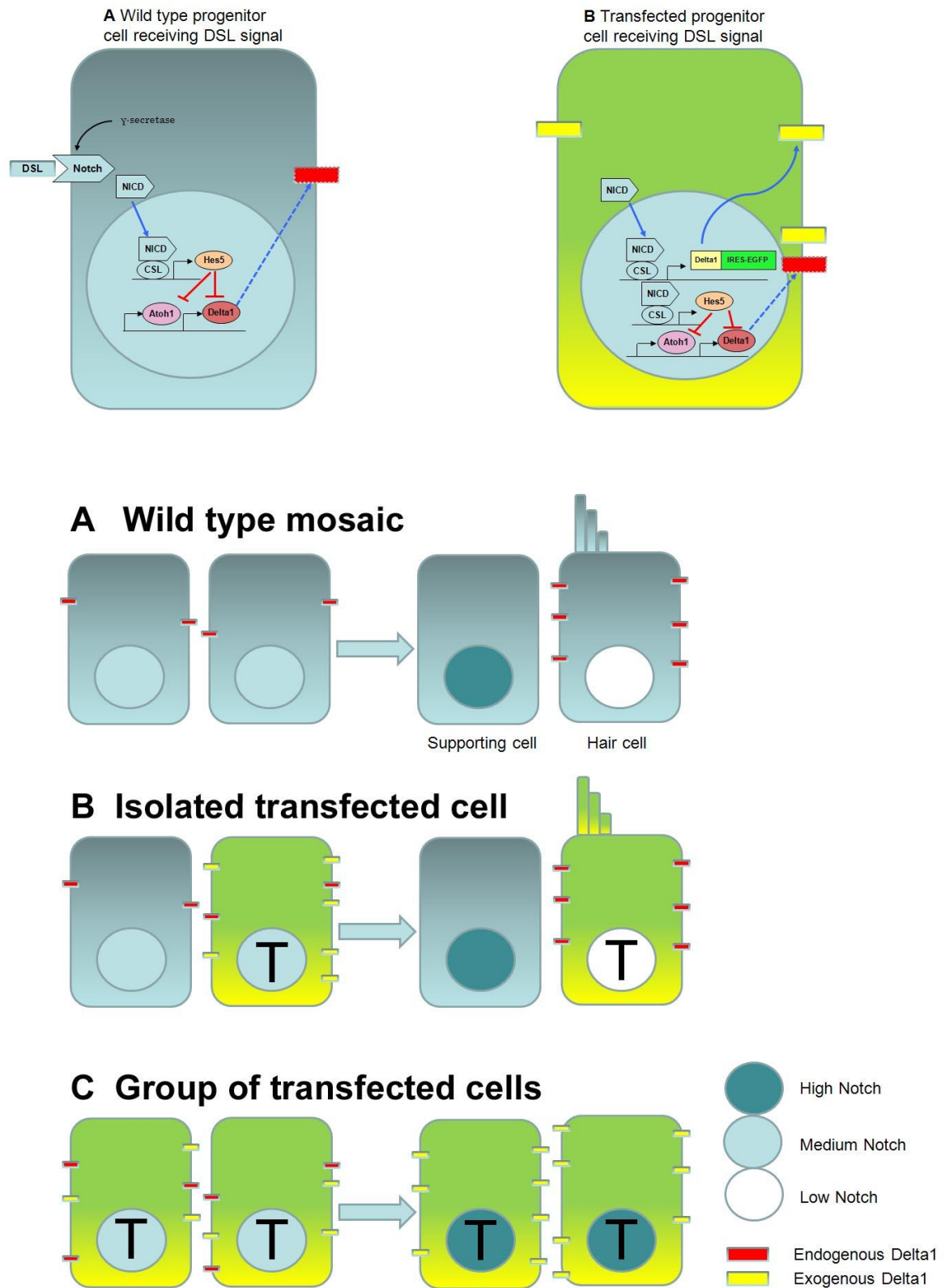
### **6.3.1 *Delta1 represses hair cell formation in trans***

My results provided further evidence supporting the central role of Delta1 in limiting hair cell formation. According to the model of lateral inhibition, it was predicted that when Delta1 is uniformly overexpressed within the sensory epithelia, all cells would be strongly delivering and receiving the inhibitory signal and hence would be prevented from differentiating into hair cells. My results fit with this prediction: there was a clear reduction in hair cell density within large patches of transfected cells. This could be observed at different stages of development within any of the sensory patches, but it was seen more clearly in the basilar papilla due to its regular arrangement of hair cells and supporting cells.

Surprisingly, Eddison et al. (2000) did not see such effect in the inner ear of chicken embryo infected with an RCAS-Delta1 construct. The reason for this unexpected result is unclear, but could possibly be explained by uncertainties about the timing of infection of analysed cells by the RCAS virus. Some of the cells exposed to ectopic Delta1 might have already committed to a hair cell fate and become refractory to any inhibitory signals. Also their analysis was performed on sections rather than whole-mounts, which may have made a slight reduction in hair cell density difficult to notice. Here, a Tol2 transposon was used to overexpress Delta1. One important difference with the RCAS vectors is that following its genomic integration, the Tol2 transposon is transmitted to the progeny of transfected cells only. Hence, cells analysed at late stages of development had the Tol2 construct throughout their developmental history. An additional advantage of using the Tol2 construct is that it gives a scattered/mosaic pattern of transfection that enables the comparison of transfected versus untransfected cells within the same region, whereas the RCAS tends to generate large patches of infection.

Unexpectedly, even highly transfected regions were not completely void of hair cells. There are several potential explanations for this observation. Firstly, it is possible that these cells had escaped Notch activity and differentiated into hair cells before their neighbours started to express

sufficiently high levels of Delta1. Once established, hair cells are thought to become ‘deaf’ to further inhibitory signals delivered by surrounding transfected cells. Secondly, my live-imaging data showed that addition of new cells and epithelial reorganisations occur at the time of hair cell production. Even within large patches of transfected cells, changes in cell position or in their levels of ectopic Delta1 expression could enable some cells to transiently ‘escape’ inhibitory signals and commit to a hair cell fate. Finally, and as I will discuss later, some form of cis-inhibition of Notch activity by DSL ligands or additional factors may have occurred in Delta1-expressing cells. Despite these uncertainties, the data show that the main effect of Delta1 is to inhibit hair cell differentiation in trans, as the standard model proposes.



**Figure 6.1 pT2K-Hes5-Delta1-IRES-EGFP mosaic transfection affects cell fate decisions: Delta1 inhibits hair cell differentiation in trans and promotes hair cell differentiation in cis. (A)** Notch-mediated lateral inhibition within sensory patches regulates hair cell differentiation **(B)** pT2K-Hes5-Delta1-IRES-EGFP isolated transfected cells (EGFP positive) upregulate Delta1 (yellow) expression within Notch active cells (sensory progenitors and supporting cells) which opposes the effect of Notch activity on endogenous Delta1 gene expression. **(C)** When Delta1 is artificially overexpressed in groups of contacting cells, all cells are strongly delivering and receiving inhibitory signal which prevents the cells from differentiating into hair cells. For simplicity Serrate1 (Jagged1 in mammals) and Serrate2 (Jagged2 in mammals) ligands are omitted from the diagram. T= Transfected with pT2K-Hes5-Delta1-IRES-EGFP.

### **6.3.2 *Delta1 is a key determinant of hair cell fate decisions***

The results also suggest that cell-to-cell competition for Delta1 expression is the mechanism responsible for the selection of the hair cell fate during lateral inhibition. In fact, I found that isolated cells that have been transfected with pT2K-Hes5-Delta1-IRES-EGFP have a greater chance of becoming hair cells than non-transfected cells. In the basilar papilla, the proportion of isolated Hes5-Delta1 transfected cells (EGFP positive cells) that differentiated into hair cells was 66%, as opposed to 30% when a control fluorescent protein was used for transfection. This result is particularly significant given that the cells in which Delta1 was ectopically expressed are those in which Notch was originally active, and should have remained as progenitors or differentiated into supporting cells. Furthermore the number of isolated transfected becoming hair cells could be underestimated, as these were no longer expressing Delta1 and EGFP at the time of analysis. Some isolated transfected cells with EGFP levels below detection threshold could have been omitted from my counts. Altogether, these results demonstrate that the capacity of progenitor cells to elevate their expression of Delta1 can promote their differentiation into hair cells, as the lateral inhibition model proposes.

### **6.3.3 *Delta1 expression is not sufficient for hair cell differentiation***

Not all of the isolated progenitor cells that were transfected differentiated into hair cells. As this analysis was done at a fixed time point, the possibility that these cells will go on to differentiate into hair cells cannot be ruled out. It is however likely that Delta1 expression in itself is not sufficient for commitment to a hair cell fate. Key determinants such as the bHLH transcription factor Atoh1 (Bermingham et al., 1999) must also be expressed. This could also explain why Delta1 upregulation did not appear sufficient to induce precocious hair cell formation either. The first hair cells in the basilar papilla appear at E6 in a circular patch at the distal end (Goodyear and Richardson, 1997). In E5-E6 transfected samples, there was no evidence of hair cell differentiation in the mid-proximal regions of the basilar papilla.

### **6.3.4 *Delta1 acts primarily in trans to regulate hair cell formation***

In ‘standard’ lateral inhibition, Delta1 expressed in the signal-sending cell activates Notch in the signal-receiving cell to inhibit its differentiation. This mechanism is referred to as ‘*trans*-inhibition’. However, studies in *Drosophila* and vertebrates suggest that Delta and Serrate can bind to Notch receptors cell-autonomously, in the signal-sending cell. If high levels



of DSL ligands are expressed at the cell surface, they can exert a cell-autonomous dominant-negative effect on Notch activity. This regulatory process, known as *cis*-inhibition, could help to restrict Notch activation to signal-receiving cells (del Alamo et al., 2011; del Alamo and Schweisguth, 2009; Ladi et al., 2005; Micchelli et al., 1997; Miller et al., 2009). In 1997, Micchelli et al., (1997) and de Celis et al. (1997) demonstrated for the first time for the first time that ligands actually display a *cis*-inhibitory effect on Notch in order to properly specify the wing margin

Evidence for *cis*-inhibition of Notch by its ligands comes from *in vivo* studies in which overexpression of DSL ligands produced phenotypes consistent with a loss of Notch activity. In *Drosophila*, high expression of DSL ligands suppress the Notch hyperactive phenotype (Heitzler and Simpson, 1993). There have also been some overexpression experiments demonstrating *cis*-inhibition in vertebrates. In *Xenopus*, Similarly to a Notch loss-of-function phenotype, an overexpression of X-Delta1 in the epidermis leads to excess differentiation of ciliated cells (Deblandre et al., 2001). In chick Delta1 and Serrate1 have been shown to reduce the activity of mouse Notch1 when co-expressed via a Hes5-luciferase reporter assay (Sakamoto et al., 2002). (For further details see del Alamo et al., 2011).

It is important to note that in these overexpression experiments, the observed effects depend on abnormally high levels of the ligands. There are however examples where *cis*-inhibition of Notch has been demonstrated by loss-of-function experiments. Ectopic Notch activation was detected in double, Serrate and Delta, mutant cells in the dorsal-ventral border of the *drosophila* wing (Micchelli et al., 1997). However, loss-of-function approaches could be disrupting both *trans*-activation and *cis*-inhibition making it difficult to show the effects of *cis*-inhibition where the two cannot be analysed separately (del Alamo and Schweisguth, 2009).

In theory, *cis*-inhibition could also be involved in promoting hair cell formation in the inner ear. It could explain why some cells that are located within transfected regions with high Delta1 expression can still differentiate into hair cells. However, if *cis*-inhibition was the predominant mechanism, it would be expected that the Hes5-Delta1 transfected regions would contain a higher hair cell density than observed. In addition, the fact that groups of Hes5-Delta1 transfected cells maintained high levels of Delta1 and EGFP expression strongly suggests that these cells do receive Notch signals efficiently. If this was not the case, the Hes5 promoter would not be

activated within such clusters. These observations suggest that cis-inhibition is either not occurring or that trans-inhibition is overriding cis-inhibition in the inner ear.

### ***6.3.5 Other mechanisms modulate Notch signalling during lateral inhibition***

The results indicate that differentiated hair cells were either GFP negative or exhibited low levels of GFP in comparison to transfected progenitors/supporting cells indicating that these cells are Notch inactive and thus have escaped Notch signalling. This is consistent with the known role for the Notch signalling pathway in maintaining the undifferentiated state of the progenitor.

During lateral inhibition, cells can ‘win’ the competition for the primary fate by sending as much inhibitory signal as possible to their neighbours, or by preventing their own reception of inhibitory signals. The latter can be a natural consequence of the intercellular feedback loop regulating the expression of DSL ligands. However, there are several possible mechanisms, in addition to cis-inactivation, that modulate the activity of the Notch pathway and that could account for the loss of Notch signalling in these cells. Each Notch molecule can transduce signal only once therefore signal strength depends on the regulation of the number and availability of Notch receptors at the cell surface. This can be regulated by different enzymes, such as O-fucosyl transferase (O-fut) that is required for the generation and possibly the removal of the receptor from the plasma membrane (Sasamura et al., 2007). E3-ligases such as, Numb, that target Notch to the lysosome for degradation (reviewed in Le Borgne (2006) could inhibit Notch signal in one of the daughters of an asymmetric mitotic division. MAM which targets NICD to poly-ubiquitination and proteasome degradation in a PEST-dependent manner could also be expressed differentially (Reviewed in Fortini et al. (2009)). These are all possible mechanism of how a progenitor cell can escape Notch to become a hair cell during lateral inhibition.

## 6.4 Conclusions

In this study, I have established new tools to examine the dynamics of Notch signalling and tested the function of Delta1 in the developing chicken inner ear. I have demonstrated that stable integration of transgenes can be achieved in the chicken inner ear using in ovo electroporation of the otic primordium with RCAN proviral DNA and Tol2 transposons.

I have shown that a reporter construct containing cis-regulatory elements of the mouse *Hes5* gene is sensitive to Notch activity in the embryonic chicken inner ear: the *Hes5*-d2EGFP reporter is active in neurosensory progenitors and robustly responds to forced activation or inhibition of Notch activity. Using live-imaging, dynamic variations in the levels of fluorescence of the reporter were found in individual progenitor cells, suggesting potential variations of endogenous Notch signalling. It was also found that extensive proliferation and cellular re-organisations occur simultaneously to hair cell differentiation within the embryonic sensory patches.

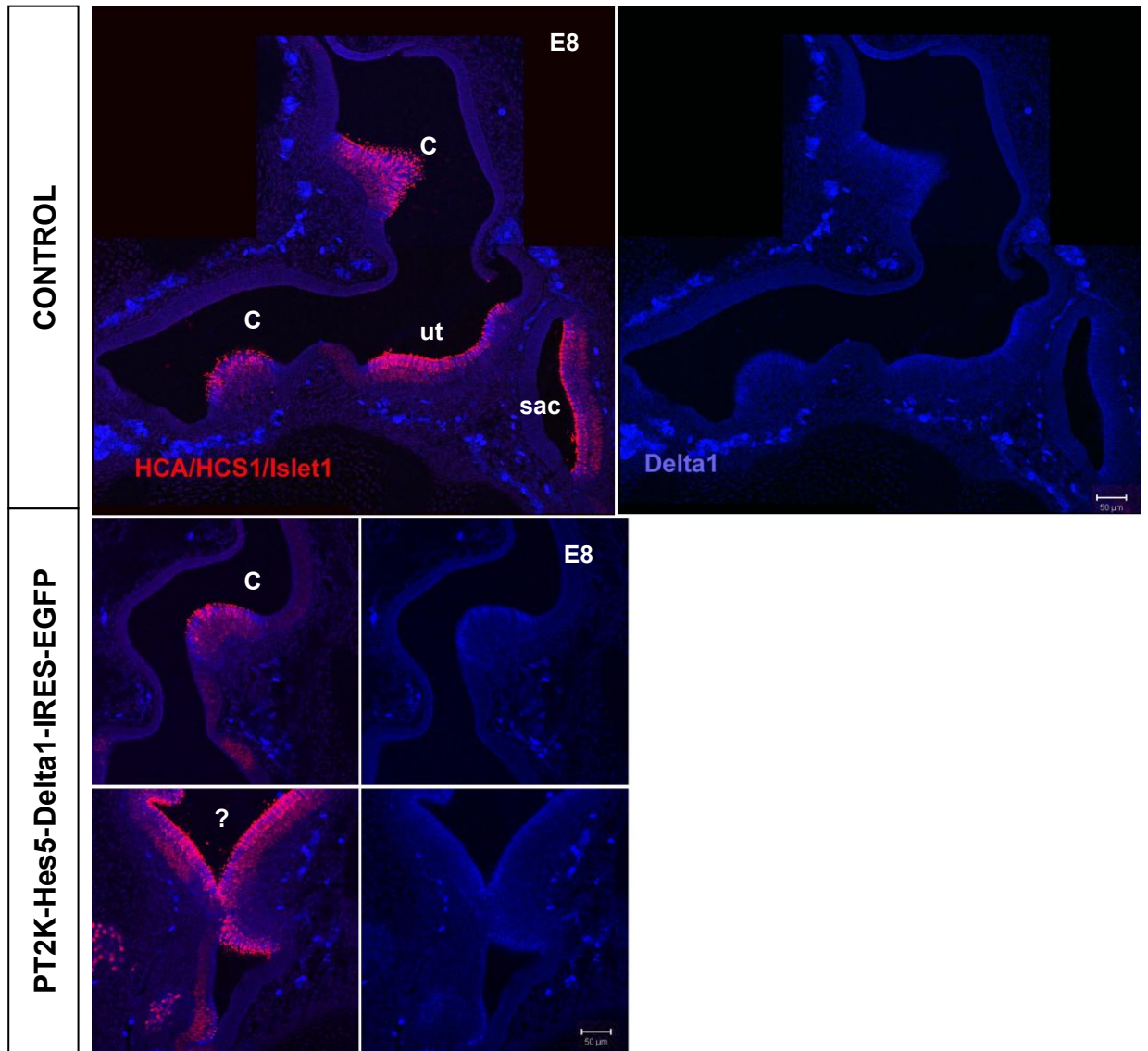
The role of Notch signalling in the early formation of the sensory epithelia is still unclear. Lateral induction, i.e. the positive regulation of *Jagged1* expression downstream of Notch activity, is thought to regulate early formation or expansion of the early sensory domains. Here I used the *Hes5* promoter region to force Delta1 expression in Notch-active cells and examined the effects of this “gain of lateral induction” on the development of the inner ear. This led to abnormal inner ear morphology as well as defects in the normal patterning of hair cells and supporting cells. The results showed that sustained Notch activity does not promote the expansion of prosensory patches, but prevents the formation of non-sensory regions in between sensory domains. This suggests that the emergence of distinct sensory epithelia from a common prosensory domain may result from the activity of localized signals that oppose lateral induction and prosensory specification.

In the course of these experiments, I also found that artificial induction of Delta1 expression affected the normal pattern of hair cell and supporting cell differentiation within sensory patches. My results showed that Delta1 acts predominantly in trans, and not in cis, during hair cell formation. The upregulation of Delta1 expression promotes, but is not sufficient for, the adoption of a hair cell fate. These data provide strong support to the proposed model of lateral inhibition with intercellular feedback.

Many questions still remain about the functions of Notch signalling during inner ear development. The dynamics of Notch signalling during hair cell formation still needs to be elucidated in order to fully understand the mechanism of hair cell formation. Do levels of Notch activity oscillate in progenitor cells before they become hair cells or is there a unidirectional decrease of Notch activity? There are still some important questions regarding the mechanism(s) for the formation of sensory domains from the initial neurosensory domain. The experiments from this study have provided some steps towards being able to answer these important questions in the field.

# 7

## APPENDIX



**PT2K-Hes5-Delta1-IRES-EGFP transfected ears have reduced or absent non-sensory domains.** An E8 ear transfected with PT2K-Hes5-Delta1-IRES-EGFP has reduced number of sensory patches indicated by hair cell markers and Islet 1 staining and reduced non-sensory regions therefore gross morphological abnormalities can be seen. c, crista; ut, utricle; sac, saccule. ? indicates unidentifiable, potentially fused patches.

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